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Health Advisory for 1,3-Dinitrobenzene

(U.S.) Environmental Protection Agency, Washington, DC

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HEALTH ADVISORY FOR 1,3-DINITROBENZENE

Criteria and Standards Division
Office of Drinking Water
U.S. Environmental Protection Agency
Washington, DC 20460

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16. ABSTRACT
This Health Advisory (HA) provides information on the health effects, analytical methodology and treatment technology that would be useful in dealing with 1,3-Dinitrobenzene contamination of drinking water. Based on available toxicity data the HA values for 1,3-Dinitrobenzene are: One-day, 0.4 mg/L; Ten-day, 0.4 mg/L; Longer-term (child), 0.4 mg/L; Longer-term (adult), 0.14 mg/L; and Lifetime, 0.001 mg/L. 1,3-Dinitrobenzene is not classifiable as to human carcinogenicity. Health Advisories describe nonregulatory concentrations of drinking water contaminants at which adverse health effects would not be anticipated to occur over specific exposure durations. The HAs, developed by the US Environmental Protection Agency, Office of Drinking Water (ODW), are not legally enforceable Federal standards and are subject to change as new information becomes available. Health Advisories are developed for One-day, Ten-day, Longer-term and Lifetime exposures based on data describing noncarcinogenic end points of toxicity. For those substances that are known or probable human carcinogens, according to the EPA classification scheme, Lifetime HAs are not recommended. The chemical concentration values for carcinogens are correlated with carcinogenic risk estimates by employing a cancer potency (unit risk) value.

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HEALTH ADVISORY FOR 1,3-DINITROBENZENE

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PREFACE

This report was prepared in accordance with the Memorandum of Understanding between the Department of the Army, Deputy for Environment Safety and Occupational Health (OASA(I&L)), and the U.S. Environmental Protection Agency (EPA), Office of Drinking Water (ODW), Criteria and Standards Division, for the purpose of developing drinking water Health Advisories (HAs) for selected environmental contaminants, as requested by the Army.

Health Advisories provide specific advice on the levels of contaminants in drinking water at which adverse health effects would not be anticipated and which include a margin of safety so as to protect the most sensitive members of the population at risk. A Health Advisory provides health effects guidelines and analytical methods, and recommends treatment techniques on a case-by-case basis. These advisories are normally prepared for One-day, Ten-day, Longer-term, and Lifetime exposure periods where available toxicological data permit. These advisories do not condone the presence of contaminants in drinking water, nor are they legally enforceable standards. They are not issued as official regulations and they may or may not lead to the issuance of national standards or Maximum Contaminant Levels (MCLs).

This report is the product of the foregoing process. Available toxicological data, as provided by the Army, on the munitions chemical 1,3-dinitrobenzene (1,3-DNB) have been reviewed, and relevant findings are presented in this report in a manner so as to allow for an evaluation of the data without continued reference to the primary documents. This report has been submitted for critical internal and external review by the EPA.

I would like to thank the authors (Dr. Nicolas P. Hajjar, Dr. Margaret E. Brower, Patricia A. Turck, Dr. Claire L. Kruger, Dr. William R. Hartley, and Dr. Welford C. Roberts) who provided the extensive technical skills required for the preparation of this report. I am grateful to the members of the EPA Tox-Review Panel who took time to review this report and to provide their invaluable input, and I would like to thank Dr. Edward Ohanian, Chief, Health Effects Branch, and Dr. Joseph Cotruvo, Director, Criteria and Standards Division, for providing me with the opportunity and encouragement to be a part of this project.

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EXECUTIVE SUMMARY

1,3-Dinitrobenzene (1,3-DNB) is a colorless to yellowish crystalline solid at room temperature. It is primarily manufactured by the nitration of nitrobenzene or benzene. It is also a predominant by-product formed during the production of nitrobenzene, TNT explosives, and nitrobenzoic acid. Release of 1,3-DNB into the aquatic environment can occur from the waste streams of dinitrobenzene and nitrobenzene production. 1,3-DNB can also be formed in the environment through the photochemical alteration of 2,4-dinitrotoluene. Small amounts of 1,3-DNB are released into the atmosphere during processing of the compound.

Microbial degradation of 1,3-DNB occurs in waste streams. Photolytic reactions may be significant; however, additional experimental data are required. Aquatic oxidation, volatilization, and hydrolysis are not known to play a significant role in the environmental fate of 1,3-DNB. Simulations predict that >99% of the compound will remain in the water column and <1% will be adsorbed to sediment. 1,3-DNB is not expected to significantly bioaccumulate in aquatic organisms.

Metabolism studies in rats and rabbits indicate that 63% to 93% of an oral dose is absorbed by the gastrointestinal tract. Absorption via the inhalation or dermal routes was inferred in humans after cyanosis, methemoglobinemia, Heinz body formation, anemia, and urinary metabolites were observed following an occupational exposure. Tissue distribution 24 hours after a single oral dose showed the highest concentrations of 1,3-DNB in the liver, white fat, brown fat, kidney, and sciatic nerve. Higher tissue residues were found in germ-free rats ($\leq$115 nmol/g) when compared with conventional rats ($\leq$5 nmol/g). Reduction of 1,3-DNB to the nitroanilines is the major metabolic pathway; 1,3-DNB is apparently not conjugated with glutathione. The principal metabolites in the urine of rabbits given an oral dose of [^{14}C]1,3-DNB are 2,4-diaminophenol, m-phenylenediamine (also known as 1,3-diaminobenzene), m-nitroaniline, and 2-amino-4-nitrophenol. In rats given an oral dose of [^{14}C]1,3-DNB, the main metabolic products were

3-aminoacetanilide, 4-acetamidophenol, 1,3-diacetamidobenzene, and 3-nitroaniline N-glucuronide. After oral administration in rats and rabbits, 1,3-DNB was primarily excreted via the urine (63 to 93%); some of the metabolites were excreted as glucuronide or sulfate conjugates. In rabbits, 1 to 5% of the radioactivity was excreted via the feces, whereas in rats 18% of the dose was excreted via the feces. In the testes 1,3-DNB appears to be nitro reduced to m-nitroaniline and m-nitroacetanilide, with m-nitrosonitrobenzene as a reactive intermediate.

In humans, the effects of 1,3-DNB intoxication after occupational exposure include cyanosis, anemia accompanied by palpitations, dizziness, and fatigue. Bilirubin and urobilinogen were seen in the urine, although no evidence of liver damage was detected. A dermal occupational exposure resulted in cyanosis and hemolytic jaundice. The metabolite 2,4-dinitrophenol was detected in the urine; formation of methemoglobin was observed in a volunteer dermally exposed to 1,3-DNB.

The oral LD_{50} values in rats are 91 mg/kg for males, 81 mg/kg for females, and 83 mg/kg for both sexes; the 95% confidence limit was 56-124 mg/kg. Toxicity in rats given a lethal dose of 1,3-DNB was characterized by a reduction in ambulatory motion, ataxia, weakness, dyspnea, rapid heartbeat, and cyanosis. Respiratory failure and coma preceded death at 1-26 hours after treatment. Older rats (105 days of age) appear to be more sensitive to the toxic effects of 1,3-DNB than younger rats (75 days of age), exhibiting mortality and neurotoxicity after a single oral dose of 48 mg/kg. No effects were seen in younger rats at this dose. Neurotoxic effects were noted following oral administration of 1,3-DNB at 20 and 25 mg/kg for one or three doses in germ-free and conventional rats, respectively. The germ-free (GF) rats were more susceptible to neurological disturbances than conventional (CV) rats following exposure to 1,3-DNB. Symptoms included ataxia with abnormal gait, flaccid paralysis of forelimbs, and splaying of hindlimbs. 1,3-DNB produced lesions of the hindbrain and cerebellum. The primary targets involved in neurotoxicity were the macroglia. Vulnerable brain regions (cerebellar roof, vestibular and superior olivary nuclei, and the inferior

colliculi) coincided with those normally displaying high rates of glucose utilization, suggesting that they may be particularly sensitive to any metabolic impairment.

The effects of a single intraperitoneal (ip) injection in rats consisted of methemoglobinemia and a reduction in sciatic motor nerve conduction velocity. Another study using a single ip dose of 1,3-DNB in rats suggested that a progressive increase in methemoglobin is associated with an increase in urinary catecholamine excretion (presumably due to enhanced plasma catecholamine levels). The plasma catecholamines accelerate hepatic glycogenolysis and hyperglycemia via an increase in phosphorylase activity. The increased rate of glycolysis due to hyperglycemia may be stimulated as a mechanism for energy production in the hypoxic state.

Both 8- and 16-week drinking water studies in rats indicated that 1,3-DNB produces splenic and testicular effects. In the 8-week study, doses of 50, 100, and 200 mg/L (5, 7, and 12 mg/kg for males; 6, 9, and 14 mg/kg for females) resulted in a significant increase in relative spleen weight in both sexes (except for males at the high dose) and a significant decrease in relative testicular weight at all dose levels. Evidence of decreased spermatogenesis was seen at the low and mid doses. At the high dose, more than half the seminiferous tubules collapsed; neither germinal nor Sertoli cells were present. Dose-related brown-yellow pigment deposition in the Kupffer cells of the liver was noted in all groups. Mortality was high at the high dose. In addition, rats of both sexes in this group showed atrophy and fibrosis of the spleen with marked hemosiderin deposition. In the 16-week study, doses of 3, 8, and 20 mg/L (0.4, 1.1, and 2.6 mg/kg for males; 0.5, 1.3, and 3.1 mg/kg for females) were administered. No effects were noted at the low dose. An increase in relative spleen weight and hemosiderin deposition was noted at the mid- and high-dose levels. At the high dose, testicular weight was significantly reduced; microscopic examination revealed a slight to moderate decrease in spermatogenesis. Based on the absence of splenic and testicular effects, the No-Observed-Adverse-Effect-Level (NOAEL) is 0.4 mg/kg/day. The Lowest-Observed-Effect-Level (LOAEL) is 1.1 mg/kg/day.

In a subchronic toxicity study in which the reproductive performance of male Sprague-Dawley rats was examined, doses of 0.75, 1.5, 3.0, or 6.0 mg/kg/day, administered by gavage 5 days/week for 12 weeks, resulted in dose-related incidences of testicular damage consisting of reduced spermatogenesis (decreases in cauda sperm reserves and testicular spermhead count) and atrophy of seminiferous tubules. Furthermore, mean litter size was significantly reduced ($p < 0.05$) at 0.75 or 1.5 mg/kg/day. Significant reductions ($p < 0.05$) in testes weight and severe testicular damage (disruption of spermatogenesis, hypo- or aspermia, significant reductions ($p < 0.05$) in cauda sperm reserves, and testicular spermhead count, and significant increases ($p < 0.05$) in abnormal sperm morphology) resulting in 100% infertility were observed in males administered 3.0 or 6.0 mg/kg/day, and no litters were produced. Dose-related increases in hemosiderin deposition in the spleen were also reported. Based on the significant reduction in litter size and dose-related incidences of testicular damage and splenic hemosiderin deposition, the LOAEL for this study was 0.75 mg/kg/day; a NOAEL was not established.

1,3-DNB has been shown to cause severe testicular damage and infertility in male rats following exposure to single oral doses. There is also some evidence to suggest that infertility resulting from a single oral exposure may not be readily reversible, thereby producing permanent testicular damage. Reduced testicular weight, tubular atrophy, disruption of germinal epithelium, loss of spermatocytes, Sertoli cell damage, and infertility have been reported in rats administered single oral doses of 15-50 mg/kg. Significant testicular damage and reduction in fertilizing ability have also been seen in rats receiving single oral doses of 5 mg/kg. Infertility was still evident in 62% of rats administered single oral doses of 48 mg 1,3-DNB/kg at 175 days after treatment. The chemical appears to act on the testes directly; the primary target of toxic action is the Sertoli cell, which functions to sustain and protect developing germ cells and regulate spermatogenesis. A single oral dose of 32 mg/kg did not change serum hypothalamic or pituitary hormone levels, indicating that 1,3-DNB does not affect hormonal control of gonadal function. Other studies have indicated that 1,3-DNB also acts independently

of follicle-stimulating hormone/cAMP (adenosine 3′,5′-cyclic phosphate) within the Sertoli cells. Age may to some extent affect the reproductive toxicity of 1,3-DNB, although systemic toxicity was also more pronounced in older animals, suggesting a possible reduction in intestinal metabolism and a subsequent increase in the absorption of parent compound in older animals. In addition, rats were found to be more sensitive to 1,3-DNB toxicity than mice; testicular damage occurred at lower doses in rats. 1,3-DNB or a metabolite appears to cause Sertoli cell vacuolization. This causes exfoliation of germinal cells from the seminiferous epithelium and ultimately results in disruption of spermatogenesis and infertility because mature sperm fail to develop. Thus, the action of 1,3-DNB appears to affect several stages of spermatogenesis by disrupting the morphology and function of Sertoli cells, but it does not appear to affect mature sperm within the epididymal lumen.

1,3-DNB has been shown to be metabolized by cultured Sertoli cells, and two major metabolites, m-nitroaniline and m-nitroacetanilide, have been identified. The toxicity of these metabolites, as well as several other proposed metabolites, including 2,4-dinitrophenol, 2-amino-4-nitrophenol, 4-amino-2-nitrophenol, and 2,4-diaminophenol, was compared to that of 1,3-DNB using Sertoli-germ cell co-cultures. Results showed that none of the metabolites tested had any effect on Sertoli cell function or morphology, indicating that 1,3-DNB can be detoxified at the target site by nitroreduction. However, another minor metabolite, m-nitrosonitrobenzene (NNB), which is proposed as an intermediate in the nitroreduction of 1,3-DNB, produced the same specific toxic effects, but at lower doses than 1,3-DNB, suggesting that 1,3-DNB may be metabolically activated and that NNB or a metabolite of NNB is the reactive intermediate in Sertoli cell toxicity.

Although 1,3-DNB has been shown by several investigators to be a potent testicular toxicant, no information on the reproductive toxicity of 1,3-DNB in females was found, and no adequate multiple-generation reproduction studies or developmental toxicity studies were available. Considering the effect observed in males, evaluation of these parameters is essential to definitively assess the reproductive and developmental toxicity of 1,3-DNB.

No lifetime studies were found in the available literature.

1,3-DNB was found to be mutagenic in Salmonella typhimurium strains TA98, TA100, TA3052, and TA1538 with and without metabolic activation, but was not mutagenic in TA1535, TA1537, and TA100 NR3 with and without metabolic activation. In additon, 1,3-DNB did not increase unscheduled DNA synthesis in cultivated hepatocytes. A toxicity study in L-cell cultures determined that 1,3-DNB reduces both protein and DNA content. Since L-cell cultures do not have the ability to metabolize 1,3-DNB by the microsomal enzyme pathway, the toxicity in the cell culture may indicate that the parent compound is responsible for damage to the testes.

Neurological effects in rats after a single lethal dose of 1,3-DNB include reduction in ambulatory motion, ataxia, and weakness. Older rats (105 days of age) appear to be more sensitive than younger rats (75 days of age), exhibiting ataxia, loss of equilibrium, and motor dysfunction after a single oral dose of 48 mg/kg. No neurotoxicity was observed in younger rats at the same dose. Neurological disturbances, including ataxia with abnormal gait, flaccid paralysis of forelimbs, and splaying of hindlimbs, were more prominent in germ-free rats than in conventional rats given two or three oral doses of 1,3-DNB. Brain lesions occurred in both groups of rats. The primary target for the damage appeared to be the macroglia. A single ip injection has been shown to decrease sciatic motor nerve conduction velocity. In rats, administration of 1,3-DNB for 12 weeks produced impaired movement, paresis, loss of equilibrium, and muscle rigidity.

In a 16-week oral toxicity study in rats, increases in splenic weight with hemosiderosis occurred at 1.14 mg/kg/day. Decreases in testicular weight also were observed and were accompanied by alterations in spermatogenesis at 2.64 mg/kg/day. However, no such effects were seen at 0.4 mg/kg/day. Based on these findings, the Longer-term Health Advisory (HA) for a 10-kg child has been determined to be 0.04 mg/L (40 µg/L). In the absence of adequate animal data to determine a One-day or Ten-day Health Advisory, the Longer-term HA for

a 10-kg child, 0.04 mg/L (40 µg/L), is used as a conservative estimate of the One-day or Ten-day HA. The Longer-term HA for an adult was established at 0.14 mg/L (14 µg/L). A Lifetime HA of 0.0010 mg/L (1.0 µg/L) for an adult was determined based on a Drinking Water Equivalent Level (DWEL) of 0.005 mg/L (5 µg/L). The DWEL is based on a Reference Dose (RfD) of 0.00013 mg/kg/day, using the same 16-week study described above for calculation of the Lifetime HA value. With respect to carcinogenic potential, 1,3-DNB is placed in Category D: not classifiable as to human carcinogenicity.

I. INTRODUCTION

The Health Advisory (HA) Program, sponsored by the Office of Drinking Water (ODW), provides information on the health effects, analytical methodology, and treatment technology that would be useful in dealing with the contamination of drinking water. Health Advisories describe nonregulatory concentrations of drinking water contaminants at which adverse health effects would not be anticipated to occur over specific exposure durations. Health Advisories contain a margin of safety to protect sensitive members of the population.

Health Advisories serve as informal technical guidance to assist Federal, State, and local officials responsible for protecting public health when emergency spills or contamination situations occur. They are not to be construed as legally enforceable Federal standards. HAs are subject to change as new information becomes available.

HAs are developed for One-day, Ten-day, Longer-term (approximately 7 years, or 10% of an individual's lifetime), and Lifetime exposures based on data describing noncarcinogenic endpoints of toxicity. For those substances that are known or probable human carcinogens, according to the Agency classification scheme (Group A or B), Lifetime HAs are not recommended. The chemical concentration values for Group A or B carcinogens are correlated with carcinogenic risk estimates by employing a cancer potency (unit risk) value together with assumptions for lifetime exposure and the consumption of drinking water. The cancer unit risk is usually derived from the linearized multistage model with 95% upper confidence limits. This provides a low-dose estimate of cancer risk to humans that is considered unlikely to pose a carcinogenic risk in excess of the stated values. Excess cancer risk estimates may also be calculated using the one-hit, Weibull, logit, and probit models. There is no current understanding of the biological mechanisms involved in cancer to suggest that any one of these models is able to predict risk more accurately than another. Because each model is based upon differing assumptions, the estimates that are derived can differ by several orders of magnitude.

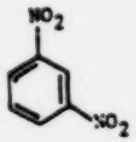
II. GENERAL INFORMATION

1,3-Dinitrobenzene (1,3-DNB) is a colorless to yellowish crystalline solid at room temperature. It is primarily manufactured by the nitration of nitrobenzene or benzene with a hot mixture of nitric and sulfuric acids. The dinitrobenzenes are also formed in small volume as by-products during the manufacture of nitrobenzene. In addition, 1,3-DNB is a by-product of the manufacture of TNT explosives at the U.S. Army ammunition plants (AAP) located at Radford, VA (Radford APP), Chattanooga, TN (Volunteer AAP), Joliet, IL (Joliet AAP), and Newport, IN (Newport AAP). It is an impurity in the final TNT product and in waste discharges (U.S. EPA, 1985).

DuPont (Deepwater, NJ) is listed as the only U.S. producer of 1,3-DNB for 1983 (more recent information is not available). Nearly all the 1,3-DNB produced by DuPont is used as an onsite intermediate in the production of m-phenylenediamine, which is used mainly as a dye intermediate. In addition, dinitrobenzenes can be used in organic synthesis and as a camphor substitute in celluloid production (U.S. EPA, 1985).

General chemical and physical properties of 1,3-DNB are presented in Table II-1.

Table II-1. General Chemical and Physical Properties of 1,3-Dinitrobenzene

CAS Number:	99-65-0	U.S. EPA (1985)
Synonyms:	m-Dinitrobenzene m-DNB 1,3-DNB	U.S. EPA (1985)
Molecular weight:	168.11	Verschueren (1983)
Empirical formula:	$C_6H_4N_2O_4$	U.S. EPA (1985)
Chemical structure:		Verschueren (1983)
Physical state:	Colorless to yellowish crystalline solid at room temperature	Verschueren (1983)
Specific gravity:	1.571 (0/4°C)	Verschueren (1983)
Melting point:	89.8°C	Verschueren (1983)
Boiling point:	300-302°C (770 mmHg)	Verschueren (1983)
Water solubility:		
@ 15°C	469 mg/L	Verschueren (1983)
@ 20°C	369 mg/L	U.S. EPA (1985)
@ 25°C	500 mg/L	U.S. EPA (1985)
@ 100°C	3200 mg/L	Verschueren (1983)
Vapor pressure:		
@ 20°C	0.0039 mmHg (extrapolated, supercooled liquid)	U.S. EPA (1985)
Log K_{ow}:	1.49	Verschueren (1983)

III. SOURCES OF EXPOSURE

The sources of exposure to 1,3-DNB have been thoroughly reviewed by U.S. EPA (1985). DuPont reportedly generates 70,000 to 72,000 pounds of 1,3-DNB annually from the production of dinitrobenzene and nitrobenzene. 1,3-DNB is also a by-product formed during the production of TNT explosive. The waste streams from these three production processes are sources of 1,3-DNB release to the aquatic environment. A concentration of 4 ppm was detected in one waste stream from TNT production (Mitchell and Dennis, 1982). It has also been estimated that at full TNT production capacity, downstream river concentrations of 1,3-DNB may range from 0.006 to 0.16 ppb. A concentration of 214 to 750 (mean = 402) mg 1,3-DNB/L was measured in wastewaters from dinitrobenzene manufacture (Swaminathan et al., 1987).

1,3-DNB can also be formed in the environment through photochemical oxidative degradation of 2,4-dinitrotoluene, a by-product released to the environment from the manufacture of TNT (Spanggord et al., 1980).

DuPont reported that approximately 60 pounds of 1,3-DNB are released into the atmosphere annually as a result of processing of the compound (TSCA-ITC, 1984, as cited in U.S. EPA, 1985).

Pertinent data regarding exposure from food and dermal contact were not found in the available literature.

IV. ENVIRONMENTAL FATE

The U.S. EPA (1985) has thoroughly reviewed the environmental fate of 1,3-DNB. In water, aromatic nitro compounds are generally resistant to hydrolysis; the chemical structure also suggests that aquatic oxidation is not significant. Photolytic reactions in the environment may be significant, but additional experimental data are required.

Mitchell and Dennis (1982) measured the microbial degradation of 1,3-DNB in waste stream samples. In nonsterilized samples, the degradation rate reached a maximum after a 10-day lag period. A sample at a concentration of 5 µg/mL was completely degraded at day 15. In autoclaved samples, no degradation was observed for a period up to 20 days. Degradation also did not occur in natural waters tested, although they contained a number of organisms comparable to those found in the waste stream samples. This suggests that the microorganisms were able to adapt to 1,3-DNB because of previous exposure.

Hallas and Alexander (1983) studied the microbial degradation of 1,3-DNB in sewage effluent maintained under aerobic or anaerobic conditions. The disappearance of 1,3-DNB under aerobic conditions was followed for 28 days; significant amounts of the ultraviolet absorbency persisted. The decrease in initial absorbance was 58% by day 28. Under anaerobic conditions, 18% of the initial absorbance was still present by day 28. Analysis by gas chromatography and combined gas chromatography-mass spectrometry showed that nitroaniline was formed from 1,3-DNB. A significant amount of the aromatic amine was formed both aerobically and anaerobically with a peak concentration at day 14 under aerobic conditions (5 µg/mL) and at day 10 under anaerobic conditions (7 µg/mL).

Microbial degradation of 1,3-DNB by Candida pulcherrima to CO_2 was demonstrated to occur under aerobic conditions (Dey and Godbole, 1986). In cultures of C. pulcherrima, m-nitrophenol, m-aminophenol, resorcinol, and fumaric acid were detected in the culture medium. No intermediate was detected between 1,3-DNB and m-nitrophenol. m-Nitrophenol was reduced to

m-aminophenol, which underwent hydrolytic deamination to resorcinol. No intermediate was detected between resorcinol and fumaric acid.

Volatilization is not significant in the environmental fate of 1,3-DNB. Experimental data regarding adsorption to sediment were not located; however, mathematical derivations from the octanol-water partition coefficient and water solubility predict that >99% of the compound will remain in the water column and <1% will be in lake sediments (EPA, 1985). Based on calculations using either the octanol-water coefficient or water solubility, 1,3-DNB is not expected to bioaccumulate significantly in aquatic organisms (EPA, 1985).

Additional information is required to evaluate the contributions of microbial degradation, chemical degradation, photolysis, adsorption, and leaching in soil with relationship to the environmental fate of 1,3-DNB. Similarly, additional information is required to evaluate the contributions of chemical degradation and photolysis in the atmospheric compartment.

V. PHARMACOKINETICS

A. ABSORPTION

Parke (1961) treated Chinchilla and English rabbits (number and sex not reported) by gavage with randomly labeled 1,3-dinitro[^{14}C]benzene in peanut oil in single doses of 50, 60, 70, or 100 mg/kg. Within 2 days, 65 to 93% of the dose was excreted in the urine and 1 to 5% was excreted in the feces. Similar results were reported for rats by Nystrom and Rickert (1987). Three male Fischer 344 rats were dosed orally with 25 mg [^{14}C]-1,3-DNB/kg; 63% of the dose was recovered in the urine, and 17% was recovered in the feces 48 hours postdosing. These studies indicate that at least 63 to 93% of an oral dose is absorbed by the gastrointestinal tracts of rats and rabbits.

Absorption of 1,3-DNB by the inhalation route in humans can be inferred from the occupational exposure studies of Okubo and Shigeta (1982) and Bresson et al. (1965). Cyanosis, methemoglobinemia, Heinz body formation, and anemia were reported as effects of exposure. Similarly, dermal absorption of 1,3-DNB can be inferred from the cyanosis and hemolytic jaundice noted after a percutaneous occupational exposure in a female worker (Ishihara et al., 1976).

B. DISTRIBUTION

The tissue distribution of [^{14}C]-1,3-DNB given as a single oral dose of 25 mg/kg to male F-344 conventional (CV) and F-344 germ-free (GF) rats was reported by Philbert et al. (1987a). All tissues taken from GF animals 24 hours after dosing had higher concentrations of radioactivity than tissues taken from CV animals; the difference in concentrations ranged from approximately ten- to twentyfold (levels of radioactivity were not tabulated; the values were estimated from a histogram). Levels of radioactivity in GF rats were highest in the liver, white fat, brown fat, kidney, and sciatic nerve (115, 83, 47, 45, and 28 nmol/g of [^{14}C]-1,3-DNB-derived equivalents, respectively). Levels of radioactivity in CV rats were 5, 1, 1, 2, and 2 nmol/g, respectively, for the same tissues. The data suggest a considerable

difference in the bioavailability (possible due to bacterial metabolism) of 1,3-DNB between the two strains.

C. METABOLISM AND EXCRETION

Two days following the oral administration of randomly labeled [^{14}C]-1,3-DNB to rabbits at doses of 50-100 mg/kg, most of the administered radioactivity (65-93% of the dose) was eliminated in the urine, and only a small amount (1-5% of the dose) was eliminated in the feces (Parke, 1961). The principal metabolites in the urine were 2,4-diaminophenol (31% of the dose), m-phenylenediamine (1,3-diaminobenzene) and m-nitroaniline (35% of the dose), and 2-amino-4-nitrophenol (14% of the dose) (Table V-1, Figure V-1). Thirty percent of an oral dose of nonradioactive 1,3-DNB was excreted as glucuronide conjugates and 6% as ethereal sulfate conjugates. Other metabolites found were 2,4-dinitrophenol (0.1%), 4-amino-2-nitrophenol (2.0%), m-nitrophenylhydroxylamine (0.8%), m-nitrosonitrobenzene (0.25%), and 3,3′-dinitroazoxybenzene (0.3%). The reduction of 1,3-DNB to m-phenylenediamine (1,3-diaminobenzene) was inhibited by aureomycin. However, Parke (1961) suggested that the intestinal microflora were not essential to the reduction of the first nitro group in 1,3-DNB. Based on the results of this study by Parke (1961), Rickert (1987) proposed a pathway for the metabolism of 1,3-DNB in rabbits (Figure V-1).

Nystrom and Rickert (1987) found that 60% of an oral dose of 25 mg/kg [^{14}C]-1,3-DNB administered to three male Fischer-344 rats, was recovered in 24 hours. Urine was the primary route of excretion, accounting for 63% of the dose at 48 hours postdosing; 18% of the radiolabel was excreted in the feces. Eight radioactive peaks were found in the sulfatase-treated urine collected 10-16 hours postdosing; all were formed through reduction. It was reported that the main metabolic pathway for 1,3-DNB was reduction followed by acetylation yielding 3-aminoacetanilide (21%), 4-acetamidophenol (7%), 1,3-diacetamidobenzene (7%), and 3-nitroaniline N-glucuronide (4%). As

Table V-1. Metabolites of [^{14}C]-1,3-DNB Recovered From the Urine and Feces of Rabbits 2 Days After Oral Administration

Compound	Average Metabolite Recovery[a]
1,3-Dinitrobenzene	0.7
m-Nitrophenylhydroxylamine	0.8
m-Nitrosonitrobenzene	0.25
3,3'-Dinitroazoxybenzene	35[b]
m-Nitroaniline	35[b]
m-Phenylenediamine	0.1
2,4-Dinitrophenol	14
4-Amino-2-nitrophenol	2.0 (0.8)[c]
2,4-Diaminophenol	31
Total metabolites	83
Total radioactivity in urine	81
Total radioactivity in feces	ND

[a]Percentage of administered dose; average of seven animals at doses between 50 and 100 mg/kg.
[b]Average of 1,3-nitroaniline and 1,3-phenylenediamine.
[c]Estimated after enzymatic hydrolysis.

ND = None detected.

SOURCE: Parke (1961).

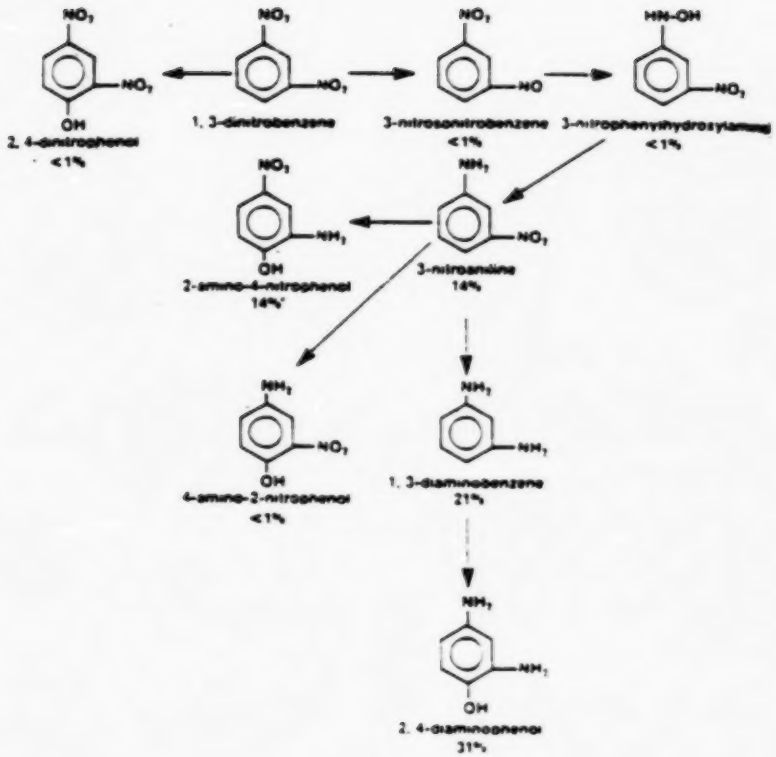


Figure V-1. Urinary metabolites of 1,3-dinitrobenzene in rabbits. Numbers are the percentage of a 50- to 100-mg/kg dose eliminated in the urine in 2 days. Pathways for the formation of secondary metabolites (e.g., 2-amino-4-nitrophenol from 3-nitroaniline) are those which seem plausible although they have not been conclusively proven.

SOURCE: Rickert (1987).

expected, 1,3-DNB was the only isomer (compared with 1,2- and 1,4-DNB) that could not be conjugated with glutathione.

Cossum and Rickert (1985) showed that isolated hepatocytes from male Fischer-344 rats were capable of reducing 1,3-DNB under aerobic conditions. Reduction was the major metabolic pathway for 1,3-DNB; m-nitroaniline accounted for 74.0 $\pm$ 1.2% of the radioactivity present after a 30-minute incubation. Incubation with microsomes produced nitroanilines as well as nitrosonitrobenzenes and nitrophenylhydroxylamines.

Cossum and Rickert (1987) investigated the conjugation of the DNB isomers with glutathione in erythrocytes from Fischer-344 rats, rhesus monkeys, and humans to determine whether conjugation occurred and, if so, whether it correlated with toxicity from methemoglobin formation. Similar to previous findings, no conjugates were formed with 1,3-DNB following incubation with erythrocytes from any of the three species. In addition, no correlation was detected between conjugation of the o- and p-DNB isomers and methemoglobin formation.

Several investigators have identified the Sertoli cell as the target for 1,3-DNB toxicity resulting in fertility problems in male rats (Blackburn et al., 1988; Foster et al., 1986; Lloyd and Foster, 1987). Foster et al. (1987) investigated the *in vitro* metabolism of 1,3-DNB and compared the *in vitro* toxicity of several metabolites, including m-nitroaniline and m-nitroacetanilide, with that of 1,3-DNB. Both Sertoli-germ cell and Sertoli cell cultures metabolized 1,3-DNB, the major route being nitroreduction. Radiolabel was incorporated into plated cells from the Sertoli-germ cell co-cultures at all doses tested (10^{-6} to 10^{-4} M [C^{14}]-1,3-DNB) to a maximum of 1% of the applied dose. Analysis of detached cells indicated incorporation of a maximum of 0.1% of the applied dose at all dose levels. The time- and dose-related appearance of radiolabel was observed in the culture medium; 24 h postexposure, a total of 6% of the radioactivity applied at 10^{-4} M was recovered. The two major metabolites identified were m-nitroaniline and m-nitroacetanilide. At least five other metabolites, comprising approximately

3% of the applied dose, were found; one was identified as the DNB-glutathione conjugate, but the others were not identified. A similar pattern of metabolism was observed following exposure to Sertoli cell cultures. Comparison of the toxicity of m-nitroaniline and m-nitroacetanilide, as well as several other metabolites (2,4-dinitrophenol, 2-amino-4-nitrophenol, 4-amino-2-nitrophenol, 2,4-diaminophenol), with 1,3-DNB revealed that none of the metabolites tested had any effect on Sertoli cell morphology or function below cytotoxic levels (10^{-4} M). Conversely, dose-related increases in germ cell loss, vacuolization of the Sertoli cell monolayer, and phagocytosis of germ cells by Sertoli cells were observed following exposure to 1,3-DNB (10^{-6} to 10^{-4} M). In a study reported by Foster (1989), m-nitrosonitrobenzene (NNB), another minor metabolite of 1,3-DNB, was found to produce morphological changes identical to that of 1,3-DNB, but at lower doses. On an equimolar basis, NNB was 3-5 times more effective than 1,3-DNB. Further work by Cave and Foster (1990) indicated that metabolic activation of 1,3-DNB is required to induce changes in Sertoli cell morphology and function, and the reactive species implicated was NNB or a metabolite of NNB. In this study, cultured rat Sertoli cells pretreated with diethyl maleate (which reduces the intracellular thiol levels), and exposed to 1,3-DNB or NNB showed enhanced sensitivity to the toxicity of both chemicals as measured by the enumeration of exfoliated germ cells. Pretreatment of cultures with cysteamine or ascorbate, which have been shown to increase intracellular thiol levels, resulted in reduced toxicity or a protective effect against 1,3-DNB and NNB toxicity. Cane and Foster (1990) proposed that within the Sertoli cell, 1,3-DNB is metabolized to m-nitroalanine and m-nitroacetanilide with NNB being a reactive intermediate; the proposed route is presented in Figure V-2.

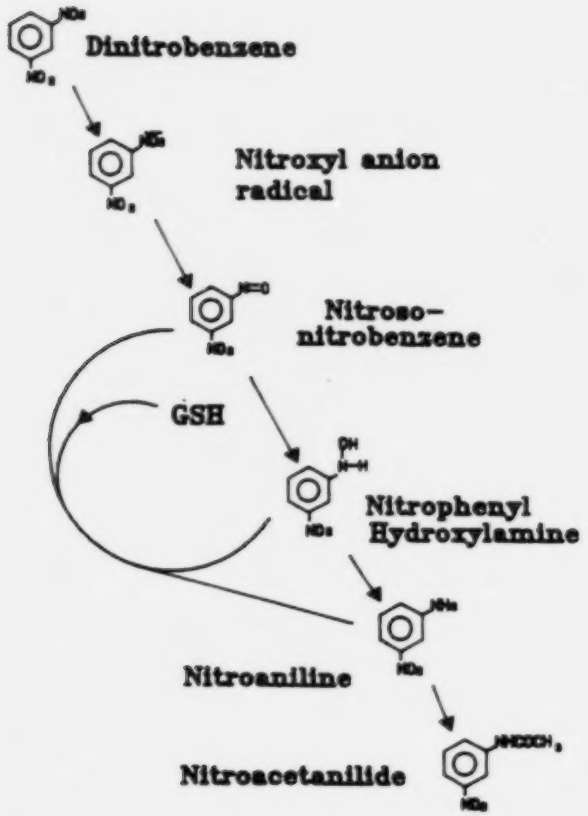


Figure V-2. Proposed route of metabolism of 1,3-dinitrobenzene by rat Sertoli cells.

SOURCE: Foster (1989).

VI. HEALTH EFFECTS

A. HUMANS

Linder et al. (1986, 1988) reported that the estimated lethal dose of 1,3-DNB in humans ranges from 5 to 50 mg/kg. In addition, humans are reported to be less resistent than rodents to methemoglobin formation as a result of comparatively less activity of methemoglobin reductase (Gleason et al., 1986). 1,3-DNB is considered to be extremely toxic to humans.

Okubo and Shigeta (1982) cited a case report of acute intoxication in six workers exposed to 1,3-DNB dust (concentration not known) who developed cyanosis within 1 day after initial exposure; the cyanosis persisted for about 2 weeks. Approximately 1 week after onset of cyanosis, the workers exhibited anemia accompanied by palpitations, dizziness, and fatigue. The magnitude of the anemia ranged from 1.9 to 15.5%, as measured by loss in specific gravity of the whole blood. Recovery from the anemia was slow; the average period was estimated to be 35 days. Other than anemia, the only significant finding was bilirubinuria and a strongly positive test for urobilinogen in the urine. No evidence of liver damage was noted by serum alanine aminotransferase and serum aspartate aminotransferase measurements. A 10-year followup through periodic health examinations did not reveal any adverse changes in the health status of the workers.

Ishihara et al. (1976) noted cyanosis and hemolytic jaundice in a female worker after percutaneous occupational exposure. Methemoglobinemia, a sign of 1,3-DNB intoxication, was not confirmed in the patient. Liver function tests were normal. Hemolytic jaundice disappeared within 10 days; the worker was considered to be practically normal within 40 days. A volunteer was subsequently exposed for a comparable period (45 minutes), under the same conditions, to the 1,3-DNB-containing (0.5% w/w solution) industrial material. The ambient air contained no 1,3-DNB. No attempt was made to quantitate the

amount absorbed; however, the presence of the urinary metabolite 2,4-dinitrophenol and the formation of methemoglobin provided evidence of 1,3-DNB absorption and intoxication.

B. ANIMAL EXPERIMENTS

1. Short-term Exposure

a. Acute

Cody et al. (1981) administered 1,3-DNB as a 1% solution in corn oil to groups of six male and six female Sprague-Dawley-derived Carworth Farms rats at one of six doses ranging from 36 to 180 mg/kg, and observed the animals observed daily for 3 weeks. In rats given a lethal dose of 1,3-DNB, toxicity was characterized by a reduction in ambulatory motion, ataxia, weakness, dyspnea, and rapid heartbeat. Paws, ears, lips, and nostrils appeared cyanotic. Eyes were protuberant, presumably congested, and dark. Respiratory failure and coma occurred as intoxication progressed; death occurred 1-26 hours after treatment. The LD_{50} was estimated to be 91 and 81 mg/kg for males and females, respectively. The corresponding 95% confidence limits were approximately 50-150 mg/kg. The LD_{50} for both sexes combined was 83 mg/kg; the 95% confidence limits were 56-124 mg/kg. For rats given a sublethal dose, the severity of the response was dose related. A temporary loss of body weight was noted; however, recovery was complete after 1 week.

Philbert et al. (1987b) studied 1,3-DNB-induced encephalopathy in a group of 15 germ-free (GF) male Fischer-344 rats given a single oral dose of 20 or 25 mg/kg and a group of 12 conventional (CV) male Fischer-344 rats given a single oral dose of 20 mg/kg. GF rats were killed at 24, 48, or 72 hours (11, 2, and 2 rats/group, respectively); CV rats were killed at 24, 48, or 72 hours (4, 4, and 4 rats/group, respectively). In addition, six CV rats received a single oral dose of 20 mg/kg/day for 3 days and were killed 72 hours after the first dose. Seven CV rats were given, by ip injection, 20 mg/kg/day for

2 days in split doses of 10 mg/kg and were killed at 48 hours. Two additional rats received a fifth dose to induce functional signs before they were killed at 54 hours. Control rats (three GF and two CV) received oral doses of polyethylene glycol 600 (PEG) (1 mL/kg) and were killed at 24 and 48 hours, respectively.

The GF rat was found to be more susceptible to 1,3-DNB than the CV rat. This was reported to be a possible function of the pharmacokinetics of 1,3-DNB, since the amount of 1,3-DNB circulating in the blood of the GF rat within 24 hours of oral dosing is >1.5 times that of the CV rat. Neurological disturbances were also more prominent among GF rats. Of 15 GF rats given a single oral dose of 1,3-DNB, 6 became ataxic; abnormal gait, flaccid paralysis of forelimbs, and splaying of hindlimbs were observed within 24 hours following dosing. An additional GF rat became slightly ataxic at 48 hours. Neurological signs were accompanied by poor feeding and weight loss. All 12 CV rats given a single oral dose of 1,3-DNB appeared to be without signs of toxicity. Five of six CV rats given three oral doses and six of nine CV rats given split ip doses were ataxic. Cyanosis was found in all rats given 1,3-DNB within 4-6 hours postdosing and persisted for 24 hours. Philbert et al. (1987b) reported that the cyanosis was due to the formation of methemoglobin following 1,3-DNB administration.

No histological lesions were seen in the 12 CV rats given a single oral dose of 20 mg/kg and killed at 24-72 hours or in 1 GF rat killed at 48 hours. Histological lesions of the hindbrain and cerebellum were found in 14/15 single-dose GF rats and 15/15 multiple-dose CV rats. The primary targets involved in the neurotoxicity of 1,3-DNB were the macroglia. Lesions noted after 48 and 72 hours in the GF rats and after 48, 54, and 72 hours in the CV rats were qualitatively similar to those noted for GF rats at 24 hours, but the severity was greater with lesions appearing more vacuolated and neurons undergoing necrosis. The authors postulated that an impairment of glucose oxidation may be associated with development of these lesions. The vulnerable brain regions (cerebellar roof, vestibular and superior olivary nuclei, and the inferior colliculi) coincide with those normally displaying a high rate of

glucose utilization, suggesting that they may be particularly sensitive to any metabolic impairment. Neurotoxic effects were observed in CV rats receiving 20 mg/kg/day as single doses over 3 days; however no effects were observed in rats receiving 20 mg/kg/day as one dose. A No-Observed-Adverse-Effect Level (NOAEL) could not be established.

Reproductive dysfunction, including marked testicular damage, was noted after a single oral dose of 1,3-DNB in rats (Blackburn et al., 1988; Rehnberg et al., 1988; Linder et al., 1988, Linder et al., 1990; Hess et al., 1988) (discussed in Section VI.3, Reproductive Effects). In a study designed to investigate the reproductive toxicity of 1,3-DNB in older versus younger adult male rats (Linder et al., 1990), age-related differences in systemic as well as reproductive toxicity were produced. Groups of male Sprague-Dawley rats, aged 75 or 105 days, were administered single oral doses of 1,3-DNB in corn oil at 0, 8, 16, 24, 32, or 48 mg/kg/day and observed for 14 days. Cyanosis was observed in rats of both ages at 16 mg/kg/day or higher. However, older rats administered 48 mg/kg/day also exhibited ataxia and loss of equilibrium. In addition, these animals displayed a type of motor dysfunction in which they held their heads to one side and continually moved in a circular motion. A total of 10/24 older high-dose animals died; no deaths or neurotoxicity were observed in younger high-dose rats. No differences in body weight or seminal vesicle, prostate, or pituitary weights were observed between control and dosed animals of either age. However, testicular weight was significantly reduced ($p < 0.05$) in both age groups at doses of 24 mg/kg/day or higher. (A further discussion of the reproductive effects observed in this study is presented in Section VI.3, Reproductive Effects.)

Pankow et al. (1975) gave groups of 10 male albino rats a single ip injection of 0.15 mmol 1,3-DNB/kg (25.2 mg/kg) as a solution in PEG M600. Methemoglobinemia was produced, causing a maximal reduction of oxygen transport capacity of about 60% by 30 minutes postinjection. Twenty-four hours after acute 1,3-DNB poisoning, the sciatic motor nerve conduction velocity was reduced by 16%. Nerve conduction returned to normal by 48 hours postinjection.

Pankow and Ponsold (1978) also studied the effect of acute poisoning with 1,3-DNB on urinary catecholamine excretion and blood sugar response in groups of 10 male albino rats injected ip with 0.15 mmol 1,3-DNB/kg (25.2 mg/kg). Methemoglobin (met-Hb) and blood sugar were measured at 5, 30, 60, 120, and 240 minutes, and 24 hours; leucine aminopeptidase (LAP) activity was measured at 24 hours postinjection. Fed rats were compared with fasted animals and bilaterally adrenalectomized animals. In a separate experiment, the urinary excretion of total catecholamines was determined in fasted rats given 0.05 (8.4 mg/kg), 0.10 (16.8 mg/kg), and 0.15 (25.2 mg/kg) mmol 1,3-DNB/kg by gavage. Urine was collected during the next 5 hours. LAP activity in serum increased in fed rats from 240 to 315 U/L ($p < 0.01$) and in fasted rats from 225 to 272 U/L ($p < 0.01$). In fed rats dosed with 0.15 mmol/kg, which produced approximately 60% met-Hb (60 minutes postinjection), an 80% increase in blood sugar concentration was detected ($p < 0.01$); however, in fasted rats, a 25% decrease was observed ($p < 0.01$). Adrenalectomized rats reacted similarly to fasted rats. With a progressive increase in met-Hb, there was an associated increase in urinary catecholamine excretion. The authors postulated that the hypoxic stress due to formation of methemoglobin evoked a release of catecholamines (presumably increasing plasma catecholamine levels), which increased urinary catecholamine excretion. The increased plasma catecholamines accelerated hepatic glycogenolysis and hyperglycemia via an increase in phosphorylase activity. The increased rate of glycolysis due to hyperglycemia may be stimulated as a mechanism for energy production in the hypoxic state.

In a study with B6C3F1 mice exposed to single oral doses of 40 and 48 mg/kg, mortality rates were 10 and 59%, respectively, in mice 11 to 19 days of age (Evenson et al., 1989a). Conversely, similar doses did not cause mortality in mice aged 26 or 85 days. A significant reduction in testes weight was observed in 85-day-old mice at 48 mg/kg. Other evidence of testicular damage seen in older but not younger animals administered 48 mg/kg included changes in the ratio of testicular cell types and chromatin structure. As seen in the study reported by Linder et al. (1990), older

animals were more severely affected than younger animals; however, mice were less susceptible to the reproductive effects of 1,3-DNB than rats (for further discussion, see Section VI.3, Reproductive Effects).

b. Primary irritation and sensitization

No information was located in the available literature.

2. Longer-term Exposure

a. 8- to 16-week studies

Cody et al. (1981) treated groups of six male and six female Carworth Farm rats for 8 weeks with 1,3-DNB in drinking water at concentrations of 50, 100, or 200 mg/L. The corresponding doses (calculated from the mean weekly water consumption values provided) were determined to be 4.74 $\pm$ 0.52; 7.26 $\pm$ 1.08, and 12.45 $\pm$ 1.63 mg/kg/day in males and 5.98 $\pm$ 0.98, 9.0 $\pm$ 1.23, and 14.44 $\pm$ 2.98 mg/kg/day in females for the low, mid, and high doses, respectively. Controls consisted of 12 males and 12 females maintained on tapwater. The mortality was high at the highest dose; 3/6 male rats died during week 4, and another died during week 5. One death occurred at week 6 and one at week 7 among female rats. A dose-related decrease in body weight gain was noted, as well as reduced food and water consumption in males and females ($p < 0.01$). Hemoglobin was significantly reduced ($p < 0.05$ to $p < 0.01$) at all dose levels in both sexes. Hematocrit values were significantly ($p < 0.01$) reduced only in females at the low and high doses. A significant ($p < 0.01$) effect on drug metabolism activity as measured by a decrease in sodium hexobarbital sleeping time in females at all dose levels and in males in the mid-dose group was seen. Increased relative splenic weight in males at the low and mid dose and in females at all dose levels ($p < 0.01$ to $p < 0.001$) and decreased testicular weight and testes-to-body weight ratio at all dose levels ($p < 0.05$ to $p < 0.001$) were observed. Histopathological examination of testicular tissue revealed evidence of decreased spermatogenesis (germinal epithelium not developing into mature sperm and a decreased number of germinal

cells) at the low and mid doses. In the high-dose group, more than half the seminiferous tubules collapsed; neither germinal nor Sertoli cells were present in the tubules. Histopathological changes in the liver consisted of brown-yellow pigment deposition in the Kupffer cells; these were least severe at the low dose and most severe at the high dose. In the high-dose group, male and female rats showed atrophy and fibrosis of the spleen with marked hemosiderin deposition. No other pathological changes were found in any of the other organs examined. A NOAEL could not be determined from this study; there were significant adverse effects on splenic and testicular weight and histopathological changes of the testes at all dose levels tested.

Cody et al. (1981) (also described in Christian et al., 1980) also administered 1,3-DNB in drinking water to groups of 20 male and 20 female Carworth Farm rats at concentrations of 0, 3, 8, or 20 mg/L for 16 weeks. The corresponding doses (calculated using a mean of the weekly water consumption values provided) were determined to be 0.40 ± 0.09, 1.14 ± 0.24 and 2.64 ± 0.64 mg/kg/day in males and 0.48 ± 0.07, 1.32 ± 0.19, and 3.10 ± 0.43 mg/kg/day in females for the low, mid, and high doses, respectively. Hematocrit and hemoglobin levels and white blood cell counts and differentials were determined for five male rats 5, 10, and 14 weeks after the start of the experiment. A record of body weight and water consumption kept throughout the study was used to determine the level of exposure to 1,3-DNB. After termination, body and organ weights were recorded, and major organs were examined histopathologically. All rats survived; no signs of acute toxicity were seen. Based on average weekly body weights, no changes were observed in growth rate of males and females in the low- and mid-dose groups or of males in the high-dose group compared with controls. Females in the high-dose group had reduced rates of growth after week 8, and average weight was significantly lower at the end of the study (263 grams as compared with 277 grams in controls). The authors concluded that the low weight was compound related, although it was partially attributable to reduced food intake. Rats at the low dose had no effects other than a possible hemosiderin deposition in the spleen; the extent of deposition was not reported. A significant ($p < 0.01$) increase in ovary weight at the low-dose level was not

present at the higher doses, suggesting that this was not a compound-related effect. Rats receiving the mid dose also may have had hemosiderin deposition in the spleen; the extent was not reported. Relative and absolute spleen weights were significantly ($p < 0.01$) in both males and females receiving the mid dose. Hexobarbital sleeping time was significantly ($p < 0.01$) prolonged in the males at this dose level. However, because there were no changes in males at the highest concentration or in females at any concentration, the authors concluded that this increase was not compound related. Hemoglobin values were moderately depressed in high-dose rats at 5 ($p < 0.05$) and 10 ($p < 0.01$) weeks; these values returned to normal from week 10 to week 14. A moderate leukopenia, evident in the control rats and in those receiving the high dose during week 10 ($p < 0.01$), was seen in all animals after 14 weeks and was not considered to be compound related. Absolute and relative splenic weights were significantly ($p < 0.001$) increased in males and females at the high dose; microscopic examination revealed a pronounced hemosiderin deposition. Testicular weights of rats in the high-dose group were significantly ($p < 0.001$) reduced; microscopic examination revealed a slight to moderate decrease in spermatogenesis. Based on effects in the spleen and testes, the NOAEL is 0.4 mg/kg/day and the Lowest-Observed-Adverse-Effect Level (LOAEL) is 1.14 mg/kg/day.

In another experiment, groups of 10 male rats were exposed to the low or mid doses (0, 3, or 8 mg/L) of 1,3-DNB in the drinking water for 90 days (Cody et al., 1981). Significantly ($p < 0.01$) increased wheel-running activity was observed during the 90 days in rats exposed to both levels of 1,3-DNB. The increase was not dose-related.

Linder et al. (1986) administered 1,3-DNB to male Sprague-Dawley rats, by gavage, 5 days/week for 12 weeks at 0, 0.75, 1.5, 3.0, and 6.0 mg/kg/day. Males were bred to untreated females during treatment week 10 and killed during treatment week 12. No effects other than slight hemosiderosis in the spleen were seen at the 0.75-mg/kg dose level. Splenic weight was increased and hemosiderosis was present at the 1.5-mg/kg dose level and above. At 6 mg/kg, decreased body weight and neurotoxic effects consisting of impaired

movement, paresis, loss of equilibrium, and muscle rigidity were noted. Based on the finding of slight hemosiderosis in the spleen, a LOAEL of 0.75 mg/kg/day was identified. No NOAEL could be established. This study is described in more detail in Section VI.3, Reproductive Effects.

b. Lifetime studies

No information was found in the available literature.

3. Reproductive Effects

Blackburn et al. (1988) investigated the nature and pathogenesis of testicular damage in male rats (Alpk/AP strain, Wistar-derived). Rats (four per group) were killed 5 days after a single oral dose of 0 or 50 mg 1,3-DNB/kg. Other groups of 40 rats received a single oral dose of 0, 5, 10, 15, or 25 mg/kg, and 8 rats/group were sacrificed at 6, 12, 24, 48, or 96 hours postdosing. A single oral dose of 50 mg/kg produced visible cyanosis within 2 hours; cyanosis was not exhibited at lower doses. Relative testicular weights were significantly reduced in rats receiving 50 mg/kg; histology revealed tubular atrophy, fragmented and vacuolated germinal epithelium, and loss of spermatocytes. Testicular lesions (Sertoli cell damage) were exhibited in animals receiving 25 mg/kg after 12 hours; Sertoli cell damage was more extensive after 24 hours. Similar effects were seen 48 hours after a single dose of 15 mg/kg. No changes in testicular morphology were observed in animals receiving 5 or 10 mg of 1,3-DNB. No changes in testicular weights were reported for animals receiving less than 50 mg/kg. Relative splenic weights were significantly increased in rats dosed with 15, 25, or 50 mg/kg; these increases were most pronounced at the highest dose. Histological examination showed that spleens appeared to be congested in these dose groups; erythropoietic activity was moderately to markedly increased at 50 mg/kg. The authors concluded that the Sertoli cell was the primary target for the toxic action of 1,3-DNB, and that germ cell damage was a secondary event. Based on changes in splenic weight and morphological changes in the testes, the LOAEL is 15 mg/kg/day and the NOAEL is 10 mg/kg/day.

Foster et al. (1986) conducted a study to identify the target cell type after a single oral dose of 1,3-DNB and to determine the effects of the compound in co-cultures of Sertoli and germ cells. Male Wistar rats received 1,3-DNB at levels of 0, 5, 10, 15, or 25 mg/kg, by gavage, and eight/group were killed at 6, 12, 24, 48, and 96 hours posttreatment. No significant decreases in testicular weight were observed at any dose of 1,3-DNB given. Light microscopic examination revealed damage to the seminiferous epithelium in rats receiving 25 mg/kg at 24 hours and in rats receiving 15 or 25 mg/kg after 48 hours. Electron microscopic examination of the testes of rats dosed at 25 mg/kg revealed cytoplasmic vacuolation and retraction of Sertoli cells in the absence of morphological changes to germ cells at 24 hours. At 48 hours, cell degeneration, necrosis, and exfoliation, particularly of late pachytene spermatocytes, occurred. Examination of Sertoli germ cell co-cultures containing 1,3-DNB at 10^{-6} to 10^{-3} M indicated that at concentrations $\geq 5 \times 10^{-6}$, the number of germ cells that had detached from the Sertoli cell monolayer increased. At concentrations up to 10^{-4}, signs of vacuolation and phagocytosis of degenerative spermatocytes were seen; at 10^{-3} M, some loss of cells from the monolayer was evident. Germ cell detachment was dose related; the authors suggested that this was not due to cytotoxicity because similar experiments (see below) measured the viability in detached cells to be from 66 to 99%. This study provides supporting evidence that the Sertoli cell is the target for testicular toxicity by 1,3-DNB.

Rehnberg et al. (1988) administered a single oral dose of 32 mg/kg 1,3-DNB to groups of eight male Sprague-Dawley rats; two rats were killed at 3 hours, 24 hours, 1 week, and 2 weeks postdosing. The effects on endocrine status were studied to ascertain whether the degenerative changes in germinal epithelium would alter the hypothalamic-pituitary axis. No effects were noted in body weights and pituitary- and androgen-dependent accessory sex organ weights (caput epididymis, seminal vesicles, and ventral and dorsolateral prostate). A significant ($p < 0.0001$) decrease in testicular weight was noted at 1 and 2 weeks postdosing. Similarly, no effects were observed in pituitary hormone concentrations or hypothalamic gonadotropin-releasing hormone except

for an increase in serum follicle-stimulating hormone (FSH) concentrations at 2 weeks. A transient decrease in serum testosterone at 24 hours returned to control values at 1 and 2 weeks. Interstitial fluid volume was increased at 2 weeks. Seminiferous tubule fluid volume was reduced within 3 hours following treatment, increased by 24 hours, and declined again over the remaining 1- and 2-week periods when compared with control levels. The reduced fluid volume from the seminiferous tubules after 3 hours indicated damage to the Sertoli cells. Testosterone concentrations in interstitial fluid and seminiferous tubules were significantly increased at 1 and 2 weeks. Androgen-binding protein concentrations in seminiferous tubule fluid were increased at 24 hours and returned to control level by 2 weeks; however, the total tubular content of androgen-binding protein was dramatically decreased at 2 weeks and remained unchanged in the caput epididymis. The concurrent increase in tubular fluid volume and androgen-binding protein concentration in the fluid is probably due to a treatment-related increase in androgen-binding protein production and/or release by the Sertoli cells. The changes in androgen-binding protein concentration were postulated to be due to an intratesticular mechanism because they occurred prior to the elevation in serum FSH. The data suggest a direct effect of 1,3-DNB on the testes and not on the hypothalamic and pituitary control of gonadal function.

In a study reported by Foster (1989), the mechanism of toxicity of 1,3-DNB on Sertoli cell function and male fertility was investigated using _in vitro_ techniques. To quantitate the effect of the chemical on the function of Sertoli cells _in vitro_, the production of lactate and pyruvate, whose secretion is thought to function in the metabolic support of germ cells, was measured after exposure of co-cultured rat Sertoli and germ cells to 1,3-DNB at concentrations of 1-10 μM (DMSO vehicle) for 24 hours. Dose-related increases in lactate and pyruvate production were observed, but 1,3-DNB had no effect on FSH-stimulated cAMP production, indicating that 1,3-DNB does not directly interfere with the function of this gonadotropin in Sertoli cells. Williams and Foster (1989) found that the addition of 1,3-DNB (>99% pure), FSH, and cAMP combined to rat Sertoli-germ cell co-cultures increased the secretion of lactate and pyruvate to a greater degree than the addition of

1,3-DNB, FSH, or cAMP alone. Thus, they concluded that 1,3-DNB acts independently of these two hormones.

Lloyd and Foster (1987) and Foster et al. (1987) investigated 1,3-DNB-produced testicular damage by studying the effect of 1,3-DNB and several putative metabolites on Sertoli-germ cell co-cultures of rat testes. [^{14}C]1,3-DNB was added to the cultures at 5 x 10^{-6} to 10^{-4} M, resulting in a significant, dose-related increase in germ cell detachment from the Sertoli cell monolayer. The viability of the detached cells varied from 96 to 66%, suggesting that the germ cell release was not due to cytotoxicity. The morphological pattern of response was analogous to that reported for 1,3-DNB in vivo; however, none of the metabolites tested [m-nitroaniline; m-phenylenediamine (1,3-diaminobenzene); 2,4-dinitrophenol; 2,4-diaminophenol; 4-amino-2-nitrophenol; and 2-amino-4-nitrophenol] exhibited this response at the concentrations tested (10^{-5} to 10^{-8}). The authors concluded that 1,3-DNB or a Sertoli cell metabolite other than those tested may be responsible for the observed testicular damage.

As part of an investigation to study the mechanism of toxicity of 1,3-DNB on Sertoli cell function (Foster, 1989), the in vitro toxicity of m-nitrosonitrobenzene (NNB), a proposed Sertoli-cell metabolic intermediate, was compared to that produced by 1,3-DNB. The addition of NNB to Sertoli cell cultures for 24 hours produced the same response (germ cell exfoliation, Sertoli cell vacuolation, presence of phagocytes within Sertoli cells) seen with 1,3-DNB, except at lower doses. On an equimolar basis, NNB was 3-5 times more effective than 1,3-DNB in producing germ cell detachment. Further work by Cave and Foster (1990) suggests that 1,3-DNB requires metabolic activation to exert its toxic effects, and that NNB or a metabolite of NNB is the reactive species.

The acute and long-term effects on sperm quality, quantity, and fertilizing ability and the histopathology of the testes were studied in male Sprague-Dawley rats following a single oral dose of 0 or 48 mg 1,3-DNB/kg (Linder et al., 1988; Hess et al., 1988). Rats (eight per group) were killed

at 1, 2, 4, 8, 16, 24, 32, 72, and 175 days; those killed at 175 days had been bred to females during weeks 3, 4, 6, 9, 13, and 24. Sluggish movements and cyanosis were noted in most treated animals; however, clinical signs of toxicity disappeared by 48 hours posttreatment. Decreased testicular and epididymal weight and testicular sperm numbers were noted by day 4; decreased cauda sperm reserves and epididymal weight occurred by days 8 and 16, respectively. By day 16, reduced numbers of motile spermatozoa and abnormal sperm morphology were seen. Fertilizing ability was slightly reduced by week 4 and declined to zero by week 6. The effects of 1,3-DNB on epididymal spermatozoa and fertilizing ability corresponded to the histologic changes in the testes. Marked damage was observed in Stages VII to XII pachytene spermatocytes at day 1 posttreatment. The regressive effects continued until day 24; after this time, the tubules either recovered or became atrophic. Using an estimate of 27 days for development from Stage VII pachytene to spermiation and 9 days for epididymal transit time, the authors calculated that the maximum effects on cauda spermatozoa would be expected at 37 days posttreatment; this agrees with the zero fertility found at days 34 to 38. The relatively mild effect on fertilizing ability observed at days 21 to 25 is inconsistent with the severe effects on cauda sperm parameters seen in nonbred animals on day 24. By day 72, most males experienced between 7 and 46% atrophic tubules; some tubules (5 to 20%) were regressive or regenerative. At 175 days, three males were normal, one had developed regressed testicles, and three males had developed atrophic tubules. This study indicates that 1,3-DNB is a potent testicular toxicant in the rat capable of producing marked testicular damage, infertility, and possible sterility from a single exposure.

Evenson et al. (1989b) studied the reproductive effects resulting from a single oral dose of 1,3-DNB to rats using flow cytometry (FCM). In this study, groups of 100-day-old male Sprague-Dawley rats (16/group) were administered, via oral gavage, 1,3-DNB in corn oil at 0, 8, 16, 32, or 48 mg/kg. Four animals/group were killed on day 1, 4, 16, or 32 posttreatment, and germinal cells from the testes, caput epididymides, and cauda epididymides were sampled from each animal to determine early and subsequent testicular changes, the numbers of abnormal cell types, and

evidence of recovery using FCM techniques. Body weight was measured on days 1, 4, 16, and 32, and testes weight was recorded on days 16 and 32 posttreatment. One day after exposure, sloughing of germinal epithelial cells into the caput epididymis and reductions in the number of pachytene spermatocytes were observed in animals administered 48 mg/kg. On day 16, alterations in the ratio of testicular cell types were observed at 32 or 48 mg/kg. This correlated with a reduction in testes weight (-12 and -41% at 32 and 48 mg/kg, respectively). Considerable recovery of normal cell-type ratios was observed at day 32, although testes weight in rats administered 48 mg/kg was still below that of controls (-19%). In addition, alteration of chromatin structure (DNA denaturation) was observed in sperm from the caput epididymis at 32 and 48 mg/kg on day 16. This suggested either direct action with sperm or an interaction with germ cells and Sertoli cells that prevented the normal development of chromatin. Changes in caput sperm chromatin were not observed on day 1 or 4 posttreatment, suggesting that 1,3-DNB did not affect sperm already in the epididymis. Alteration of chromatin structure persisted in rats administered 48 mg/kg to day 32. Also observed were dose-related increases in spermhead abnormalities within the cauda epididymis and vas deferens. Based on the results of this study, the NOEL for reproductive toxicity was 16 mg/kg in rats.

Similar results were seen in a study designed to detect reduction in fertility using in vitro fertilization techniques (Holloway et al., 1990). Groups of 9 or 12 male Wistar rats, weighing 475-625 g, received single oral doses of 5, 15, or 25 mg 1,3-DNB/kg in polyethylene glycol 600 at a volume of 1 mL/kg. In addition, a group of 33 male rats was administered the vehicle only and served as controls. Fertility was assessed using in vitro techniques twice a week for 12.5 weeks, and sperm velocity measurements and light microscopy and electron microscopy examinations of testes were performed at specified time points. Administration of 15 or 25 mg/kg resulted in severe disruption of germinal epithelium with exfoliation of germ cells between 0.5 and 3.5 weeks and after 4.5 weeks posttreatment. In seminiferous epithelium that still had germ cells present, disruption of spermatogenesis continued to be evident. Significant testicular damage was also observed in 5/9 low-dose

animals. A subsequent, significant reduction in sperm-fertilizing capacity was seen at 1.5 to 5 and 7.5 to 8.5 weeks posttreatment in mid- and high-dose animals and at 3, 5.5, 7.5, and 8.5 weeks posttreatment in low-dose animals. These results suggested that all Sertoli cells were not equally affected, but that the effect occurred in a stage-specific fashion. The incidence and severity of testicular lesions and reduced fertility occurred in a dose-related pattern; the most severe effects occurred at 25 mg/kg. Also observed were significant reductions in sperm velocity 1 week after treatment in the mid- and high-dose groups. After 4 weeks, less than 20% of sperm recovered from these animals were motile. Based on the testicular damage observed at both doses tested, the LOEL was 5 mg/kg, and a NOEL was not established.

In a study designed to evaluate the influence of testicular maturation on the reproductive toxicity observed following single oral doses of 1,3-DNB in male rats, Linder et al. (1990) found that older animals were more sensitive to 1,3-DNB exposure than younger animals. In this study, groups of male Sprague-Dawley rats, aged 75 or 105 days, were administered single oral doses of 1,3-DNB (>98% pure) in corn oil at 0, 8, 16, 24, 32, or 48 mg/kg and observed for 14 days. These animals were killed after the observation period, and testes, epididymides, prostates, seminal vesicles, and pituitaries were weighed. Sperm motility, total count, and concentration were measured, and sperm morphology was examined using phase contrast microscopy. Furthermore, testes were examined histologically, and spermatogenesis was evaluated. Additional groups of rats received single oral doses of 0 or 48 mg/kg. These animals were then mated to untreated females at 4 days, 5 weeks, and 5 months posttreatment to evaluate fertility. After mating was confirmed, eggs were collected from females and scored as fertilized (eggs containing two pronuclei and a sperm tail or meiotic chromosomes and a condensed spermhead with associated sperm tail) or unfertilized (no sperm tail).

The high dose proved to be excessively toxic to older animals; a mortality rate of 42% and neurotoxicity were observed. Conversely, no mortality or neurotoxicity was seen in the younger animals at 48 mg/kg. Reproductive toxicity was also more pronounced at lower dose levels in older

animals. Testicular weight was significantly reduced ($p < 0.05$) at 24 mg/kg or greater in both age groups. Significant reductions ($p < 0.05$) in testicular spermhead count were observed at 24 mg/kg or greater in younger animals and 16 mg/kg or greater in older animals. Histological examination revealed dose-related increases in testicular damage; greater severity was observed in older animals than in younger animals. Morphological changes in the testes consisted of loss of early spermatids and some tubules with reduction in the number of late spermatids, seminiferous tubules with disorganized epithelium and retention of late spermatids at the lumina and basal aspects of the germinal epithelium, and the appearance of some deformed late spermatid nuclei and occasional multinucleated giant cells. At 24 mg/kg or greater, seminiferous tubular atrophy and reductions in pachytene/diplotene spermatocytes were observed.

Significant reductions ($p < 0.05$) in epididymal weight and total numbers of spermatozoa in the cauda epididymis were observed at 24 mg/kg or greater in younger animals and 16 mg/kg or greater in older animals. Sperm concentration in the cauda luminal fluid was reduced at 32 and 48 mg/kg in younger animals and at 24 and 32 mg/kg, but not at 48 mg/kg in older animals. In the distal cauda epididymis, abnormal spermatozoa, primarily decapitated or otherwise degenerated cells, were observed at 24 mg/kg or greater in younger rats and 16 mg/kg or greater in older rats. At 48 mg/kg in older animals, these epididymal changes were not as pronounced. Luminal debris and round bodies were observed, although the epithelium was intact in both age groups at 16 mg/kg. At higher doses, hypospermia was observed in the caput, corpus, and cauda epididymis, and round bodies were distributed throughout the cauda. At 48 mg/kg in older animals, aspermia of the caput epididymis was observed; few or no round bodies were seen. In the proximal and midcauda, hypospermia and round bodies were observed, and in the distal cauda, many normal-appearing sections filled with normal sperm were found at 48 mg/kg. In general, the amount of testicular debris was less than that found in animals at lower doses with less severe damage. Sperm motility was significantly reduced ($p < 0.01$) at 24 mg/kg or greater, although the effect was not as discernible (significant at $p < 0.05$) at 48 mg/kg in older animals.

At 5 weeks posttreatment, 91% (10/11) younger high-dose animals were infertile, and in older high-dose animals, 37% were infertile and 63% (5/8) were subfertile (<80% eggs fertilized). One week later, rebreeding of subfertile animals revealed that three had become infertile. By 5 months posttreatment, only three high-dose animals (two younger and one older) were infertile. Approximately 6 months after dosing, rats receiving 1,3-DNB had not fully recovered, although remaining effects were minimal according to the study authors. Improvement in sperm production, storage, and quality were observed in the majority of dosed animals. However, tubular atrophy remained in all dosed animals, although incidence was 2% or less in 45 and 62% of the younger and older rats, respectively. Other changes included occasional incidences of disorganized epithelia and retention of condensed spermatid nuclei and round bodies in the epididymis. Numerous atrophic tubules and reduced or incomplete spermatogenesis were observed in dosed animals that failed to regain fertility.

Sperm concentration, motility, and morphology appeared to be less affected in older animals receiving 48 mg/kg than in lower dose groups, although severe testicular effects were observed. These findings correlated with histological evidence of pockets of normal sperm in the most distal parts of the caudal epididymis. The authors postulated that this was due to a change in the movement of luminal elements in the epididymis, which resulted from an interruption in the normal flow of tubular fluid. This in turn may have resulted from a decrease in the amount of fluid in the seminiferous tubules or from neurological inhibition of the peristaltic movement in the epididymis preventing fluid flow, since 1,3-DNB has been shown to cause neurotoxicity. Based on the results of this study, the NOEL and LOEL for reproductive effects were 8 and 16 mg/kg, respectively.

In a study conducted to assess the reproductive effects of 1,3-DNB using FCM techniques in prepubertal, pubertal, and adult mice, Evenson et al. (1989a) found that mice treated similarly to rats were less susceptible to the toxic effects of single oral doses of 1,3-DNB. Also, as reported by other

investigators in studies with rats (Linder et al., 1990), the effects noted in adult mice were more pronounced than those observed in younger mice, indicating that age may affect the degree of testicular damage. In this study, groups of 13 male B6C3F1 mice, 26 or 85 days of age, were administered, via gavage, single oral doses of 1,3-DNB (in corn oil/acetone vehicle) at 0, 8, 16, 32, or 48 mg/kg. In addition, groups of 16 mice (11 to 19 days old) were similarly treated with 0, 40, or 48 mg/kg. On day 1, 3, 11, or 22 posttreatment (time points corresponding to the time points evaluated in rats), two to four pubertal or adult mice were killed, and testes, epididymides, and vas deferens were excised and processed for evaluation using FCM techniques. Groups of three to four prepubertal mice were killed at various time points after treatment, and reproductive organs were evaluated as described above. A dose of 48 mg/kg resulted in a mortality rate of 59% in prepubertal mice; however, at 40 mg/kg, 90% of the mice survived. Data from the survivors of these two groups were pooled for comparison with controls. Results indicated that the testes weight of adults was significantly reduced ($p < 0.05$) on day 22 posttreatment, but not at other time points. No germinal epithelial cells were found in the epididymis at any dose level at any time point in mice. The change in the ratio of testicular cell types (elongated and round spermatids) was significant in adult mice administered 48 mg/kg, but similar changes were not observed in prepubertal or pubertal mice at this dose. A reduction in tetraploid cells was observed in high-dose adult mice on day 1 posttreatment, suggesting that they are the primary target of 1,3-DNB toxicity. Administration of 1,3-DNB appeared to magnify the abnormal chromatin structure that is generally observed upon initiation of sperm production in pubertal mice. In high-dose adult mice, significant increases in abnormal chromatin structure were seen on day 11 posttreatment ($p < 0.05$) in the caput epididymis and on day 22 posttreatment ($p < 0.01$) in the cauda epididymis. From the results of this study, the NOEL for reproductive effects in adult mice was 32 mg/kg, which compares to a NOEL of 16 mg/kg in rats. The species difference may be due to a slower rate of metabolism of 1,3-DNB to more reactive forms in mice (Gleason et al., 1969). For prepubertal and pubertal mice, the NOEL for reproductive effects appears to be 48 mg/kg, although this dose proved to cause excessive systemic toxicity in prepubertal mice.

Foster (1989) investigated the effect of repeated oral administration of 1,3-DNB on male fertility. Sexually mature male rats (proven fertile) were administered oral doses of 5 or 10 mg 1,3-DNB/kg/day (in polyethylene glycol 600) for 5 days, and then continually mated with untreated females for 8 weeks. Groups of males were killed during and after the mating period; mated females were killed, and uterine contents were examined 15-19 days after mating was confirmed. At 10 mg/kg/day, testes weight was significantly reduced ($p < 0.05$) just prior to mating and at every time point ($p < 0.05$ or 0.01) examined during the mating period. Testes weight was also significantly reduced at 5 mg/kg/day prior to mating ($p < 0.05$) but not at all the examined time points during mating; by 9 weeks posttreatment, testes weight of low-dose animals was similar to that of controls. At 5 mg/kg/day, reductions in fertility were observed during the mating period, but the number of pregnant females was comparable to that of controls at week 9. Reductions in numbers of implants and live fetuses as well as changes in other fertility parameters (not reported) were observed in rats administered 1,3-DNB. At 10 mg/kg/day, significant reductions in number of pregnant females were observed during weeks 3-9. In a group of high-dose males mated after 16 weeks, fertility was comparable to that of control animals. Effects on spermatocytes and spermatids similar to those observed by Blackburn et al. (1988) were noted following histopathological evaluation of males killed during the beginning of the mating period (dose levels not specified). At the end of the mating period, however, normal spermatogenesis to the level of spermatocyte/early spermatid development was found, but further maturation was not evident. Consequently, reductions in fertility were due to the lack of development of mature sperm and not to preimplantation loss. No dominant lethal effect was found.

The effect on testicular toxicity and infertility of 1,3-DNB to male Sprague-Dawley rats administered, by gavage, doses of 0, 0.75, 1.5, 3.0, and 6.0 mg/kg/day, 5 days/week for 12 weeks, was investigated by Linder et al. (1986). Males were bred to untreated females during week 10 and killed during

week 12. Dose-related reductions in spermatogenesis and atrophy of seminiferous tubules, as well as dose-related increases in the incidence of splenic hemosiderin deposition, were noted. Slight reductions in cauda sperm reserves (5%) and testicular spermhead count (9%) were observed at 0.75 mg/kg/day, and significant reductions ($p < 0.05$) were observed in these parameters at 1.5 mg/kg/day. Average litter size was significantly decreased ($p < 0.05$) at both the 0.75- and 1.5-mg/kg levels. Slight decreases in the number of implantation sites and slight increases in resorptions were also noted at 0.75 and 1.5 mg/kg/day. Females mated to males in the 3-mg/kg group were confirmed sperm positive; however, no implantation sites were seen. Significant ($p < 0.05$) alterations in sperm morphology, spermhead count, and cauda sperm reserves in the males from this group were observed. The seminiferous tubules were atrophic, and no normal spermatogenesis was seen. Testicular and epididymal weights were significantly ($p < 0.05$) decreased. Rats dosed at 6 mg/kg exhibited decreased body weight gain and slight ataxia at week 8. The neurotoxic effects observed at 6 mg/kg, particularly during mating, consisted of impaired movement, paresis, loss of equilibrium, and muscle rigidity. A mean weight loss of 71 g (16%) occurred during the 1-week breeding period; two males died. Twelve males from the 6-mg/kg group were mated to two females each; insemination was confirmed in only one female, and no implantation sites were observed. Decreased testicular and epididymal weights ($p < 0.05$) were seen. Hypospermia and aspermia were noted. No motile sperm were found. After a 5-month recovery period, some alterations in spermatogenesis persisted. This study demonstrated that subchronic exposure to relatively low levels of 1,3-DNB can produce effects on the testes. Spermatogenesis at all dose levels was adversely affected; this was reflected in reductions of cauda sperm reserves, testicular spermhead count, and implantations; increased number of resorptions; and significant reductions in litter size at 0.75 mg/kg or higher. The study authors considered the significant decreases in litter size to be equivocal; however, the corresponding changes in other reproductive parameters indicated that this was a treatment-related finding. Infertility was noted at 3 and 6 mg/kg. At 3 mg/kg, infertility appeared to be due to decreased sperm quantity and quality. At 6 mg/kg, the authors postulated that a combination of

neurological effects and aspermic ejaculates may have been responsible for insemination failure. The antispermatogenic effects appeared to be at least partially reversible. Based on the significant reductions in litter size and dose-related reductions in cauda sperm reserves and testicular spermhead count, as well as the finding of slight hemosiderosis in the spleen, a LOAEL of 0.75 mg/kg/day was identified. A NOAEL could not be established.

4. Developmental Toxicity

No information was found in the available literature.

5. Carcinogenicity

No information was found in the available literature.

6. Genotoxicity

As summarized in Table VI-1, 1,3-DNB was mutagenic in Salmonella typhimurium strains TA98 and TA100 with and without metabolic activation (Spanggord et al., 1982; McGregor et al., 1980; Kawai et al., 1987). Chiu et al. (1978) found 1,3-DNB to be mutagenic in TA98 but not in TA100; evaluation was made at only one log dose interval with only three concentrations tested without metabolic activation. 1,3-DNB was mutagenic in TA1538 with and without metabolic activation (Spanggord et al., 1982; McGregor et al., 1980; Garner and Nutman, 1977). Spanggord et al. (1982) found that 1,3-DNB was mutagenic in TA98 and TA100 but was not mutagenic in TA1535, TA1537, and TA100 NR3 (nitroreductase negative strain) with or without S9. McGregor et al. (1980) found only slight activity in TA 1537 and no mutagenicity in TA1535.

In addition to the studies summarized in Table VI-1, Probst et al. (1981) reported that 1,3-DNB did not increase unscheduled DNA synthesis in cultivated hepatocytes at concentrations from 0.5 to 1,000 nmoles/mL. It was, however, positive for reverse mutations in S. typhimurium strains TA1538, TA98, TA100,

Table VI-1. Genotoxicity of 1,3-DNB

Indicator organism	Dose range	Activation	Comments	Reference
		Gene Mutation		
Salmonella typhimurium TA100, TA98	62.5, 125, 250, 500, 1,000 µg/plate	± S9	Mutagenic in TA100 and TA98 both with and without S9. Response stronger with S9. The minimum effective dose estimated from a graph was 125 µg/plate for TA98 and 250 µg/plate for TA100.	Kawai et al. (1987)
S. typhimurium TA1535, TA1537, TA1538, TA98, TA100, TA100 NR3	Range from 0 to 600 µg/plate	± S9	Negative in TA1535, TA1537, TA100NR3 with and without S9. Mutagenic in TA1538, TA98, TA100 with and without S9. The minimum effective dose estimated from a graph was 250 µg/plate for TA100.	Spanggord et al. (1982)
S. typhimurium TA1537, TA1538, TA98, TA100, TA1535	1, 3.3, 10, 33.3, 100, 333.3, 1,000 µg/plate	± S9	Negative in TA1535 with and without S9. Mutagenic in TA1538 TA98, TA100 with and without S9. Slight activity in TA1537. S9 mix reduced the magnitude of the responses. The minimum effective dose level was 33.3 µg/plate.	McGregor et al. (1980)

(continued)

Table VI-1. (cont.)

Indicator organism	Dose range	Activation	Comments	Reference
S. typhimurium TA98, TA100	0.1, 1.0, 10 µmol/plate	- S9	Mutagenic in TA98, negative in TA100. The minimum effective dose level was 0.1 nmol/plate. Bacterial reduction of one or both nitro groups may have produced a compound that was responsible for the mutagenicity in TA98.	Chiu et al. (1978)
S. typhimurium TA1538	50, 100 µg/plate	± S9	Mutagenic both with and without S9. The minimum effective dose level was 50 mg/plate.	Garner and Nutman (1977)
		Mitotic Gene Conversion		
Saccharomyces cerevisiae D5	Range up to 32 mg/mL	± S9	No indications of recombinogenic activity or any other type of genetic toxicity in presence or absence of S9.	McGregor et al. (1980)
		DNA Repair		
Escherichia coli W3110/polA+, p3478/polA-	0.1, 0.5, 1.0, 5.0 and 10.0 mg/plate	± S9	Toxicity occurred at all dose levels with and without S9; however, no differential toxicity between the plates containing E. coli polA+ vs. polA-.	McGregor et al. (1980)

and D3052 tested over a 10,000-fold concentration range with and without metabolic activation; the minimal mutagenic concentration was 0.6 mmoles/mL.

Cody et al. (1981) conducted seven experiments to determine the concentration-related response of L-cell cultures (a continuous line of mouse connective tissue cells) to 1,3-DNB at dosages ranging from 0.1 to 100 mg/L. A standard deviation of less than 15% of the mean protein content was used as the criterion for acceptability of the experiment. The concentration causing a 50% reduction in protein content (LC_{50}) and the concentration causing a 20% mean reduction in protein content (minimum-effect concentration) were determined. The response to 1,3-DNB based on DNA content was the same as that based on protein content; both protein content and DNA content were reduced in the same proportion. At 3 and 8 mg/L, protein and DNA were decreased to roughly 85% of control. At 100 mg/L, both DNA and protein were approximately 5% of control. Microscopic observations of morphological changes in treated cultures and vital staining with erythrosine B showed that cell losses at higher concentrations were due primarily to cell death and destruction and not to detachment of healthy cells from the surface of the tissue culture flask. At lower concentrations where there was incomplete cell death, excessive granularity of the cytoplasm and loss of characteristic fibroblastic shape were noted to be concentration dependent.

Cultures of L-cells do not have aryl hydrocarbon hydroxylase activity and may also lack the ability to attack 1,3-DNB by other microsomal enzyme pathways. The authors postulated that the toxicity demonstrated in the cell culture may indicate that unmetabolized 1,3-DNB could be responsible for cell damage to the testicles.

7. Neurotoxicity

As indicated in several studies described in the previous sections, 1,3-DNB is neurotoxic. Acute toxicity after a single lethal dose of 1,3-DNB in rats was characterized by a reduction in ambulatory motion, ataxia, and weakness (Cody et al., 1981). A single oral dose of 48 mg/kg resulted in

ataxia and loss of equilibrium in Sprague-Dawley rats, 105 days of age (Linder et al., 1990). These animals also exhibited a type of motor dysfunction in which they held their heads to one side and moved in a continuous circular pattern. Younger rats, 75 days of age, treated similarly, did not exhibit any neurotoxic symptoms. After single or multiple oral doses of 1,3-DNB, encephalopathy was produced in GF and CV rats (Philbert et al., 1987b). Neurological disturbances were more prominent among GF rats; symptoms included ataxia with abnormal gait, flaccid paralysis of the forelimbs, and splaying of the hindlimbs. Light and electron microscopic examination revealed brain stem lesions frequently associated with petechial hemorrhages. The primary cellular target appeared to be the macroglia. Pankow et al. (1975) noted a decrease in sciatic motor nerve conduction velocity 24 hours after a single ip injection of 1,3-DNB. Neurotoxic effects consisting of impaired movement, paresis, loss of equilibrium, and muscle rigidity were observed after administration of 1,3-DNB for 12 weeks by gavage (Linder et al., 1986).

A study reported by Cody et al. (1981) indicating significant elevations in wheel-running activity at 3 and 8 mg/L 1,3-DNB in drinking water was not considered to be an acceptable study. The results of this study were discussed with Dr. Joan Cranmer, a well-known neurobehaviorist at the University of Arkansas. The test was run over a 90-day period and all results were combined into one figure, thus ignoring the considerations of biological rhythms and estrous cycles. No dose-related effect was seen. In addition, no significant effect was found for the second behavioral test (activity platform counts); the results from the two tests should have been correlated. Also, more than two doses should have been used.

VII. HEALTH ADVISORY DEVELOPMENT

A. SUMMARY OF HEALTH EFFECTS DATA

In humans, the major toxic effect of exposure to 1,3-DNB is cyanosis; the onset ranged from 1 day to several weeks after initial exposure. Other reported effects include methemoglobinemia and anemia (Okubo and Shigeta, 1982; Bresson et al., 1965; Ishihara et al., 1976).

An acute toxicity study by Cody et al. (1981) indicated that the oral LD_{50} in rats was 91, 81, and 83 mg/kg for males, females, and both sexes combined, respectively. Toxicity in rats given a lethal dose of 1,3-DNB was characterized by a reduction in ambulatory motion, ataxia, weakness, dyspnea, rapid heartbeat, and cyanosis. Respiratory failure and coma preceded death at 1-26 hours posttreatment. There is some evidence suggesting that older rats are more susceptible to the toxic effects of 1,3-DNB than younger rats. In a study by Linder et al. (1990), excessive mortality (42%) and neurotoxicity (ataxia, loss of equilibrium, and motor dysfunction) were observed in older rats, 105 days of age, administered single oral doses of 48 mg 1,3-DNB/kg, but not in younger rats, 75 days of age, receiving the same dose. Conversely, prepubertal mice (11-19 days old) administered 48 mg/kg showed increased mortality (59%) compared to older animals (26 or 85 days old) receiving the same dose (Evenson et al., 1989a). Philbert et al. (1987b) demonstrated brain lesions in GF and CV rats given one to three oral doses of 20 or 25 mg 1,3-DNB/kg. Petechial hemorrhages were frequently associated with the lesions. Neurological disturbances were more prominent among GF rats. The primary targets involved in the neurotoxicity were the macroglia. The vulnerable brain regions coincided with those normally displaying a high rate of glucose utilization, suggesting that they may be particularly sensitive to metabolic impairment.

In rats, a single ip injection of 25.2 mg 1,3-DNB/kg was shown to produce methemoglobinemia and reduction in nerve conduction velocity (Pankow et al., 1975); another study using an ip injection of 25.2 mg 1,3-DNB/kg demonstrated

that increased methemoglobin is associated with an increase in urinary catecholamine excretion (Pankow and Ponsold, 1978). The increased catecholamines were postulated to be the result of an increased rate of glycolysis produced in response to the hyperglycemia used as a mechanism for energy production in the hypoxic state.

An 8-week study in rats given 50, 100, or 200 mg 1,3-DNB/L in drinking water (5-6, 7-9, and 12-14 mg/kg, respectively, in males and females) indicated effects in the spleen and testes (Cody et al., 1981). Increased relative spleen weight occurred at all dose levels in both sexes, and decreased testicular weight was observed at all dose levels. Decreased spermatogenesis at the low and mid doses was indicated by the lack of development of the germinal epithelium into mature sperm and by a decreased number of germinal cells. At the high dose, more than half of the seminiferous tubules collapsed with no germinal cells or Sertoli cells present. Brown-yellow pigment deposition was seen in the Kupffer cells of the liver at all doses. Rats at the high dose also exhibited atrophy and fibrosis of the spleen with marked hemosiderin deposition. Marked mortality was seen in this group.

A 16-week study in rats given 0, 3, 8, or 20 mg 1,3-DNB/L in drinking water (0.4, 1.1, and 2.6 mg/kg to males; 0.5, 1.3, and 3.1 mg/kg to females) produced no effects at the low dose (Cody et al., 1981; Christian et al., 1980). In both the mid- and high-dose groups, increased relative spleen weight and hemosiderin deposition were seen; these were pronounced at the high dose. Testicular weights were significantly reduced, and microscopic examination revealed a slight to moderate decrease in spermatogenesis in the high-dose group.

No data were available on the carcinogenicity of 1,3-DNB; no lifetime studies were found in the available literature.

1,3-DNB has been showed to produce marked testicular damage and infertility in male rats following a single oral dose. The Sertoli cells have

been implicated as the primary target with germ cell damage as a secondary event. Single doses of 15, 25, or 50 mg/kg 1,3-DNB produced testicular damage as evidenced by reduced testicular weight (at 50 mg/kg only), tubular atrophy, disruption of germinal epithelium, loss of spermatocytes, and Sertoli cell damage (Blackburn et al., 1988). Furthermore, single oral doses at 5 mg/kg resulted in significant testicular damage in 56% of treated rats (Holloway et al., 1990). Possible sterility, as well as testicular damage and infertility, were observed in rats administered a single oral dose of 48 mg 1,3-DNB/kg (Linder et al., 1988). Failure to observe changes in hypothalamic or pituitary hormone concentrations after a single dose of 32 mg 1,3-DNB/kg indicated that the chemical's effect on the testes may be direct and not the result of an alteration in the hypothalamic and pituitary control of gonadal function (Rehnberg et al., 1988). Further work by Williams and Foster (1989) indicated that 1,3-DNB also acts independently of follicle-stimulating hormone and cAMP within the Sertoli cell.

Foster (1989) investigated the effect of repeated oral exposure on male reproduction and concluded that the infertility resulting from administration of 5 or 10 mg/kg/day for 5 days appeared to be due to the lack of development of mature sperm owing to the absence of Sertoli cell support. Sixteen weeks after treatment, however, fertility was restored to control levels, demonstrating that infertility, in this case, was not permanent. Oral doses ranging from 1.5 to 6.0 mg/kg/day given 5 days per week for 12 weeks produced decreased testicular and epididymal weights, marked testicular damage with damage to the seminiferous tubules, and alterations in spermatogenesis. Decreased fertility was observed at 3.0 and 6.0 mg/kg. No effects were noted at 0.75 mg/kg.

In a subchronic toxicity study in which the reproductive performance of male Sprague-Dawley rats was examined (Linder et al., 1986), doses of 0.75, 1.5, 3.0, or 6.0 mg/kg/day, administered by gavage 5 days/week for 12 weeks, resulted in dose-related incidences of testicular damage consisting of reduced spermatogenesis (decreases in cauda sperm reserves and testicular spermhead count) and atrophy of seminiferous tubules. Furthermore, mean litter size was

significantly reduced ($p < 0.05$) at 0.75 or 1.5 mg/kg/day. Significant reductions ($p < 0.05$) in testes weight and severe testicular damage (disruption of spermatogenesis, hypo- or aspermia, significant reductions ($p < 0.05$) in cauda sperm reserves, testicular spermhead count, and significant increases ($p < 0.05$) in abnormal sperm morphology) resulting in 100% infertility were observed in males administered 3.0 or 6.0 mg/kg/day, and no litters were produced. Dose-related increases in hemosiderin deposition in the spleen were also reported. Based on the significant reduction in litter size and dose-related incidences of testicular damage and splenic hemosiderin deposition, the LOAEL for this study was 0.75 mg/kg/day; a NOAEL was not established.

Studies using Sertoli germ cell co-cultures provided additional evidence that the Sertoli cell is the target for 1,3-DNB toxicity. Several possible metabolites of 1,3-DNB, including m-nitroaniline, 2,4-dinitrophenol, m-nitroacetanilide, and 2-amino-4-nitrophenol did not produce any effects when exposed to the co-cultures. However, another minor metabolite, n-nitrosonitrobenzene (NNB), produced the same effects seen following 1,3-DNB exposure, but at low doses, suggesting that 1,3-DNB may require metabolic activation and that NNB or a metabolite of NNB is the reactive intermediate in Sertoli cell toxicity.

1,3-DNB was mutagenic to S. typhimurium strains TA98 and TA100 with and without metabolic activation (Spanggord et al., 1982; McGregor et al., 1980; Kawai et al., 1987); it was also mutagenic in TA1538 with and without metabolic activation (Spanggord et al., 1982; McGregor et al., 1980; Garner and Nutman, 1977). 1,3-DNB was not mutagenic in TA1535, TA1537, and TA100 NR3 with and without activation (Spanggord et al., 1982).

Concentrations of 0.1 to 100 mg 1,3-DNB/L in L-cell cultures resulted in a reduction of DNA and protein concentration. Cell toxicity was characterized by excessive granularity of the cytoplasm and loss of characteristic fibroblastic shape (Cody et al., 1981). Since L-cell cultures do not have the ability to metabolize 1,3-DNB by the microsomal enzyme pathway, toxicity to

the culture may indicate that 1,3-DNB and not a metabolite is responsible for Sertoli cell damage.

Neurological effects in rats after a single lethal dose of 1,3-DNB include reduction in ambulatory motion, ataxia, weakness, and motor dysfunction. Age was shown to affect neurotoxicity with older rats (105 days of age) being more sensitive than younger rats (75 days of age) (Linder et al., 1990). Neurological disturbances, including ataxia with abnormal gait, flaccid paralysis of forelimbs, and splaying of hindlimbs, were more prominent in GF rats than in CV rats given two or three oral doses of 1,3-DNB. Brain lesions occurred in both groups of rats (Philbert et al., 1987b). The primary target for the damage appeared to be the macroglia. Single ip injection has been shown to decrease sciatic motor nerve conduction velocity (Pankow et al., 1975). Oral administration of 1,3-DNB for 12 weeks to rats produced impaired movement, paresis, loss of equilibrium, and muscle rigidity (Linder et al., 1986).

B. QUANTIFICATION OF TOXICOLOGICAL EFFECTS

Health Advisories are generally determined for One-day, Ten-day, Longer-term (approximately 7 years), and Lifetime exposures if adequate data are available that identify a sensitive noncarcinogenic endpoint of toxicity. The HAs for noncarcinogenic toxicants are derived using the following formula:

$$HA = \frac{(\text{NOAEL or LOAEL})(bw)}{(UF)\ (____\ L/day)} = ____\ mg/L\ (____\ \mu g/L)$$

where:

NOAEL or LOAEL = No- or Lowest-Observed-Adverse-Effect Level (in mg/kg bw/day).

bw = assumed body weight of a child (10 kg) or an adult (70 kg).

UF = uncertainty factor (10, 100 or 1,000), in accordance with NAS/ODW guidelines.

L/day = assumed daily water consumption of a child (1 L/day) or an adult (2 L/day).

1. One-day Health Advisory

No data found in the available literature were suitable for determination of the One-day HA value for 1,3-DNB. It is therefore recommended that the Longer-term HA for a 10-kg child (0.04 mg/L) be used as a conservative estimate for the One-day HA value.

2. Ten-day Health Advisory

No data found in the available literature were suitable for determination of the Ten-day HA value for 1,3-DNB. It is therefore recommended that the Longer-term HA for a 10-kg child (0.04 mg/L) be used as a conservative estimate for the Ten-day HA value.

3. Longer-term Health Advisory

In a 16-week study, male and female Sprague-Dawley-derived Carworth Farm rats received 0, 3, 8, or 20 mg 1,3-DNB/L in drinking water (Cody et al., 1981). These doses correspond in males and females, respectively, as follows: 0.40 $\pm$ 0.09 and 0.48 $\pm$ 0.07; 1.14 $\pm$ 0.24 and 1.32 $\pm$ 0.19; 2.64 $\pm$ 0.64 and 3.10 $\pm$ 0.43 mg/kg/day. No effects were reported at 3 mg/L (0.4 mg/kg/day in males), other than a slight hemosiderin deposition in the spleen. Although the extent of deposition was not reported, it was stated that this effect was pronounced only at the highest dose. Absolute and relative spleen weights were significantly ($p < 0.01$, $p < 0.05$) increased in males and females dosed with 8 and 20 mg/L. Testicular weights were significantly reduced in males receiving 20 mg/L; a slight to moderate decrease in spermatogenesis was exhibited in this group. Based on splenic and testicular effects, the LOAEL is 1.14 mg/kg/day. The NOAEL is 0.4 mg/kg/day based on the absence of splenic and testicular effects.

The use of 0.4 mg 1,3-DNB/kg/day as the NOAEL is supported in the study by Linder et al. (1986) who administered 0, 0.75, 1.5, 3.0, and 6.0 mg/kg/day to male Sprague-Dawley rats by oral gavage, 5 days/week for 12 weeks. At

0.75 mg/kg/day, the only effects noted were slight hemosiderosis in the spleen and a significant decrease in litter size. At 1.5 mg/kg/day, additional effects seen included a significant increase in spleen weight and a significant decrease in spermhead count. The authors noted that although smaller live litters were produced at these dose levels, a dose-response relationship was not apparent and the litter sizes were well within the normal limits for the rat strain used. Therefore, this effect on fecundity was considered equivocal.

The Longer-term HA for the 10-kg child is calculated as follows:

$$\frac{(0.4 \text{ mg/kg/day})\ (10 \text{ kg})}{(1 \text{ L/day})\ (100)} = 0.04 \text{ mg/L } (40\ \mu\text{g/L})$$

where:

0.4 mg/kg/day = NOAEL, based on a lack of effect on spleen and testicular weights with no histological changes in the testes or alterations in spermatogenesis.

10 kg = assumed weight of a child.

1 L/day = assumed water consumption of a 10-kg child.

100 = uncertainty factor, chosen in accordance with ODW/NAS guidelines using a NOAEL from an animal study.

The Longer-term HA for a 70-kg adult is calculated as follows:

$$\frac{(0.4 \text{ mg/kg/day})\ (70 \text{ kg})}{(2 \text{ L/day})\ (100)} = 0.14 \text{ mg/L } (140\ \mu\text{g/L})$$

where:

0.4 mg/kg/day = NOAEL, based on a lack of effect on spleen and testicular weights with no histological changes in the testes or alterations in spermatogenesis.

70 kg = assumed weight of an adult.

2 L/day = assumed water consumption of a 70-kg adult.

100 = uncertainty factor, chosen in accordance with ODW/NAS guidelines using a NOAEL from an animal study.

4. Lifetime Health Advisory

The Lifetime HA represents that portion of an individual's total exposure that is attributed to drinking water and is considered protective of noncarcinogenic adverse health effects over a lifetime exposure. The Lifetime HA is derived in a three-step process. Step 1 determines the Reference Dose (RfD), formerly called the Acceptable Daily Intake (ADI). The RfD is an estimate of a daily exposure to the human population that is likely to be without appreciable risk of deleterious effects over a lifetime, and is derived from the NOAEL (or LOAEL), identified from a chronic (or subchronic) study, divided by an uncertainty factor(s). From the RfD, a Drinking Water Equivalent Level (DWEL) can be determined (Step 2). A DWEL is a medium-specific (i.e., Drinking water) lifetime exposure level, assuming 100% exposure from that medium, at which adverse, noncarcinogenic health effects would not be expected to occur. The DWEL is derived from the multiplication of the RfD by the assumed body weight of an adult and divided by the assumed daily water consumption of an adult. The Lifetime HA is determined in Step 3 by factoring in sources of exposure, the relative source contribution (RSC). The RSC from drinking water is based on actual exposure data or, if data are not available, a value of 20% is assumed. If the contaminant is classified as a Group A or B carcinogen, according to the Agency's classification scheme of carcinogenic potential (U.S. EPA, 1986), then caution should be exercised in assessing the risks associated with lifetime exposure to this chemical.

No data found in the available literature were suitable for determination of the Lifetime HA value for 1,3-DNB. It is therefore recommended that the 16-week study by Cody et al. (1981) described above be used to derive the 70-kg adult Lifetime HA value. Testicular weights of rats in the high-dose group (2.64 mg/kg/day) were significantly reduced ($p<0.001$); microscopic examination revealed a slight to moderate decrease in spermatogenesis. The LOAEL is 1.14 mg/kg/day, based on possible hemosiderin deposition and a significant increase in relative spleen weight. The NOAEL is 0.4 mg/kg/day based on the absence of testicular and splenic effects.

Step 1. Determination of the Reference Dose (RFD)

$$RFD = \frac{(0.4\ mg/kg/day)}{1{,}000 \times 3} = 0.00013\ mg/kg/day\ (0.1\ \mu g/kg/day)$$

where:

0.4 mg/kg/day = NOAEL, based on a lack of effect on spleen and testicular weights with no histological changes in the testes or alterations in spermatogenesis.

1,000 = uncertainty factor, chosen in accordance with ODW/NAS guidelines (10x for intraspecies variability, 10x for interspecies variability, and 10x for use of a NOAEL from a subchronic study for a lifetime value).

3 = uncertainty factor for the lack of subchronic and reproductive toxicity data.

Step 2: Determination of the Drinking Water Equivalent Level (DWEL)

$$DWEL = \frac{(0.00013\ mg/kg/day)\ (70\ kg)}{2\ L/day} = 0.0046\ mg/L\ (5.0\ \mu g/L)$$

where:

0.00013 mg/kg/day = RfD.

70 kg = assumed body weight of an adult.

2 L/day = assumed daily water consumption of an adult.

Step 3: Determination of a Lifetime Health Advisory

$$Lifetime\ HA = (0.0046\ mg/L)\ (0.2) = 0.0009\ mg/L\ (1.0\ \mu g/L)$$

where:

0.0046 mg/L = Drinking Water Equivalent Level (DWEL).

0.2 = assumed Relative Source Contribution (RSC) if actual data are not available.

C. QUANTIFICATION OF CARCINOGENIC POTENTIAL

Applying the criteria described in EPA's guidelines for assessment of carcinogenic risk (U.S. EPA, 1986), 1,3-DNB is classified in Group D: not classified as to human carcinogenicity.

VIII. OTHER CRITERIA, GUIDANCE, AND STANDARDS

The American Conference of Governmental Industrial Hygienists (ACGIH, 1990) adopted a Time Weighted Average-Threshold Limit Value (TWA-TLV) of 1 mg/m^3 (0.15 ppm) for exposure to dinitrobenzene (all isomers). The Occupational Safety and Health Administration (OSHA) standard for exposure to dinitrobenzene (all isomers) in the workplace requires that the concentration not exceed 1 mg/m^3 on an 8-hour TWA basis (CFR, 1981). Both the OSHA standard and the ACGIH criterion are followed by a notation emphasizing the importance of the cutaneous route of exposure and including mucous membranes and eyes in the absorption of 1,3-DNB.

IX. ANALYTICAL METHODS

Methods for analyzing trace quantities of 1,3-DNB include gas chromatography (GC), electron capture, tandem mass spectrometry (MS), and spectrophotometric determination.

For GC, samples were extracted in methylene chloride. The concentrations detected from 1,3-DNB wastewater ranged from 214 to 750 µg/mL (mean = 402 µg/mL) (Swaminathan et al., 1987). Concentrations detected in river samples downstream from a munitions plant ranged from approximately 5.25 to 0.125 µg/mL (Mitchell and Dennis, 1982). At 1,3-DNB concentrations below 1 µg/mL, a nitrogen-phosphorus detector was used and ethyl acetate was substituted for methylene chloride as the extracting solvent.

Another method for the determination of 1,3-DNB at levels as low as 0.1 µg/L used resin extraction followed by elution with ethyl acetate, concentration of the eluate, separation by capillary GC, and detection using electron capture (Richard and Junk, 1986).

An MS method used to detect and identify 1,3-DNB incorporates two stages, with the first acting as a separator for the second, and an analyte-specific detector. For this study, three detection methods were used to determine the most suitable one. Electron impact ionization is least suitable for the analysis; positive and negative chemical ionization methods, however, generate the types of ions most useful for analysis by tandem mass spectrometry (McLuckey et al., 1985).

X. TREATMENT TECHNOLOGIES

Hallas and Alexander (1983) studied the microbial degradation of 1,3-DNB in sewage effluent maintained under aerobic or anaerobic conditions. The disappearance of 1,3-DNB under aerobic conditions was followed for 28 days; significant amounts of the ultraviolet absorbency persisted. The decrease in initial absorbance was 58% by day 28. Under anaerobic conditions, 18% of the initial absorbance was still present by day 28. Analysis by gas chromatography and combined gas chromatography-mass spectrometry showed that nitroaniline was formed from 1,3-DNB. A significant amount of the aromatic amine was formed both aerobically and anaerobically with a peak concentration at day 14 under aerobic conditions (5 μg/mL) and at day 10 under anaerobic conditions (7 μg/mL).

Microbial degradation of 1,3-DNB by Candida pulcherrima to CO_2 was demonstrated to occur under aerobic conditions (Dey and Godbole, 1986). In cultures of C. pulcherrima, m-nitrophenol, m-aminophenol, resorcinol, and fumaric acid were detected in the culture medium. No intermediate was detected between 1,3-DNB and m-nitrophenol. m-Nitrophenol was reduced to m-aminophenol, which underwent hydrolytic deamination to resorcinol. No intermediate was detected between resorcinol and fumaric acid.

Simmons and Zepp (1986) reported that photolysis of 1,3-DNB occurs in water. The degradation of 1,3-DNB was enhanced by dissolved humic substances.

Treatment of wastewater generated in the production of dinitrobenzene by slaking with lime, followed by removal of the organics contained in the lime in a muffle furnace, was investigated by Deshmukh et al. (1984). A 1:1 ratio of wastewater:lime was determined to be optimum. Lime added to wastewater at the optimum ratio was considered to be the best mode of mixing; results indicated that more water was removed this way because of a better utilization of the heat generated during slaking. The optimum temperature for destroying organic impurities was 300°C. At this temperature, 99.1% of the chemical oxygen demand (a measure of organic content) was removed.

XI. CONCLUSIONS

Based on the available animal data and on adverse effects on the spleen and testes of rats administered 1,3-DNB by oral gavage for 16 weeks, the Longer-term HA for a 10-kg child has been determined to be 0.04 mg/L (40 µg/L). In the absence of adequate animal data to determine a One-day or Ten-day HA, the Longer-term HA for a 10-kg child, 0.04 mg/L (40 µg/L), is used as a conservative estimate of the One-day or Ten-day HA. The Longer-term HA for a 70-kg adult was determined to be 0.14 mg/L (140 µg/L). A Lifetime HA of 0.0010 mg/L (1.0 µg/L) for a 70-kg adult has been determined, based on a Drinking Water Equivalent Level (DWEL) of 0.005 mg/L (5 µg/L). The DWEL is based on a Reference Dose of 0.00013 mg/kg/day. The Reference Dose (RfD) was calculated from a NOAEL of 0.4 mg/kg/day which was based upon the absence of splenic and testicular effects in rats fed 1,3-DNB in the diet for 16 weeks.

Since no chronic toxicity or carcinogenicity studies with 1,3-DNB are currently available, 1,3-DNB is classified in Group D: not classifiable as to human carcinogenicity.

A companion report "Data Deficiencies/Problem Areas and Recommendations for Additional Data Base Development for 1,3-DNB" (see appendix) summarizes the scope of existing data reviewed for this HA. This companion report delineates the areas where additional data and/or a clarification of existing data would be appropriate for a sound HA.

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APPENDIX

Data Deficiencies/Problem Areas and Recommendations for Additional Data Base Development for 1,3-DNB

DATA BASE DEVELOPMENT

A. OBJECTIVES

The objective of this document is to provide an evaluation of data deficiencies and/or problem areas encountered in the review process for 1,3-dinitrobenzene (1,3-DNB) and to make recommendations, as appropriate, for additional data base development. This document is presented as an independent analysis of the current status of 1,3-DNB toxicology, as related to its possible presence in drinking water, and includes a summary of the background information used in development of the Health Advisory (HA). For greater detail on the toxicology of 1,3-DNB, the Health Advisory on 1,3-DNB should be consulted.

B. BACKGROUND

1,3-DNB is a colorless to yellowish crystalline solid at room temperature. It is manufactured predominantly by the nitration of nitrobenzene or benzene. It is also a predominant by-product formed during the production of nitrobenzene, TNT explosives, and nitrobenzoic acid (U.S. EPA, 1985). Release of 1,3-DNB into the aquatic environment can occur from the waste streams of dinitrobenzene and nitrobenzene production. 1,3-DNB can also be formed in the environment through the photochemical alteration of 2,4-dinitrotoluene. Small amounts of 1,3-DNB are released into the atmosphere during processing of the compound.

In water, microbial degradation of 1,3-DNB occurs in waste streams (Mitchell and Dennis, 1982). Photolytic reactions may be significant; however, additional experimental data are required. Aquatic oxidation, volatilization, and hydrolysis are not thought to play a significant role in the environmental fate of 1,3-DNB. Simulations predict that >99% of the compound will remain in the water column and <1% will be adsorbed to sediment. 1,3-DNB is not expected to significantly bioaccumulate in aquatic organisms (U.S. EPA, 1985).

Metabolism studies in rats and rabbits indicate that 63 to 93% of an oral dose is absorbed by the gastrointestinal tract (Parke, 1961; Nystrom and Rickert, 1987). Absorption via the inhalation or dermal routes was inferred in humans after detection of cyanosis, methemoglobinemia, Heinz body formation, anemia, and urinary metabolites following occupational exposure (Okubo and Shigeta, 1982; Bresson et al., 1965; Ishihara et al., 1976). Tissue distribution 24 hours after a single oral dose showed the highest concentrations of 1,3-DNB in the liver, white fat, brown fat, kidney, and sciatic nerve (Philbert et al., 1987a). Higher tissue residues were found in germ-free rats ($\leq$115 nM/g) when compared with conventional rats ($\leq$5 nM/g).

Reduction of 1,3-DNB to the nitroanilines is the major metabolic pathway; 1,3-DNB is apparently not conjugated with glutathione. The principal metabolites in the urine of rabbits given an oral dose of [^{14}C]1,3-DNB are 2,4-diaminophenol, 1,3-phenylenediamine, 1,3-nitroaniline, and 2-amino-4-nitrophenol (Parke, 1961). In rats given an oral dose of [^{14}C]1,3-DNB, the main metabolic products were 3-aminoacetanilide, 4-acetamidophenol, 1,3-diacetamidobenzene, and 3-nitroaniline-N-glucuronide (Nystrom and Rickert, 1987). In vitro studies suggest that within the testicular Sertoli cell, 1,3-DNB is metabolized via nitroreduction to m-nitroaniline and m-nitroacetanilide, with m-nitrosonitrobenzene being a reactive intermediate (Foster et al., 1987; Foster, 1989; Cave and Foster, 1990). The primary route of excretion for 1,3-DNB after oral administration in rats and rabbits is the urine (63 to 93%), with some of the metabolites excreted as glucuronide or sulfate conjugates. In rabbits, 1 to 5% of the radioactivity was excreted in the feces (Parke, 1961), whereas in rats, 18% of the dose was excreted in the feces (Nystrom and Rickert, 1987).

In humans, the effect of 1,3-DNB intoxication after occupational exposure was the onset of cyanosis, anemia accompanied by palpitation, dizziness, fatigue, and languor. Bilirubin and urobilinogen in the urine were noted, although no evidence of liver damage was seen (Okubo and Shigeta, 1982). A dermal occupational exposure resulted in cyanosis and hemolytic jaundice.

The metabolite 2,4-dinitrophenol was found in the urine, and formation of methemoglobin was detected in a volunteer dermally exposed to 1,3-DNB (Ishihara et al., 1976).

The oral LD_{50} values in rats are 91 mg/kg for males, 81 mg/kg for females, and 83 mg/kg for both sexes with 95% confidence limits of 56-124 mg/kg (Cody et al., 1981). Toxicity in rats given a lethal dose of 1,3-DNB was characterized by a reduction in ambulatory motion, ataxia, weakness, dyspnea, rapid heartbeat, and cyanosis. Respiratory failure and coma preceded death at 1-26 hours after treatment. Older rats (107 days) appeared to be more susceptible to the toxic effects of 1,3-DNB than younger rats (25 days of age), exhibiting mortality and neurotoxicity after a single oral dose of 48 mg/kg (Linder et al., 1990). No effects were observed in younger rats given the same dose. Neurotoxic effects were noted following oral administration of 1,3-DNB at 20 or 25 mg/kg for one or three doses in germ-free (GF) or conventional (CV) rats. The GF rats were more susceptible to neurological disturbances following administration of 1,3-DNB than the CV rats. Symptoms included ataxia with abnormal gait, flaccid paralysis of forelimbs, and splaying of hindlimbs. 1,3-DNB produced lesions of the hindbrain and cerebellum. The primary targets involved in neurotoxicity were the macroglia. Vulnerable brain regions coincided with those normally displaying high rates of glucose utilization, suggesting that they may be particularly sensitive to any metabolic impairment (Philbert et al., 1987b).

The effect of a single ip injection in rats was methemoglobinemia and a reduction in sciatic motor nerve conduction velocity (Pankow et al., 1975). Another study using a single ip dose of 1,3-DNB in rats suggested that with a progressive increase in methemoglobin, there is an associated increase in urinary catecholamine excretion. The catecholamines accelerate hepatic glycogenolysis and hyperglycemia via an increase in phosphorylase activity. The increased rate of glycolysis due to hyperglycemia may be stimulated as a mechanism for energy production in the hypoxic state (Pankow and Ponsold, 1978).

Both 8- and 16-week drinking water studies in rats indicated that 1,3-DNB produces effects on the spleen and the testes. In the 8-week study, doses of 50, 100, and 200 mg/L (5, 7, and 12 mg/kg for males; 6, 9, and 14 mg/kg for females) resulted in a significant increase in relative splenic weight in both sexes (except for males at the high dose), and a significant decrease in relative testicular weight at all dose levels (Cody et al., 1981). At the low and mid doses, there was evidence of decreased spermatogenesis. At the high dose, more than half the seminiferous tubules collapsed; neither germinal cells nor Sertoli cells were present. Brown-yellow pigment deposition in the Kupffer cells of the liver was present in all groups and was dose related. Mortality was high at the high dose. In addition, rats of both sexes in this group showed atrophy and fibrosis of the spleen with marked hemosiderin deposition. In the 16-week study, doses of 3, 8, and 20 mg/L (0.4, 1.1, and 2.6 mg/kg for males; 0.5, 1.3, and 3.1 mg/kg for females) were used. No effects were noted at the low dose (Cody et al., 1981; Christian et al., 1980). An increase in relative spleen weight and hemosiderin deposition was noted at the mid and high doses. At the high dose, testicular weight was significantly reduced; microscopic examination revealed a slight to moderate decrease in spermatogenesis.

No lifetime studies were found in the available literature.

1,3-DNB has been shown to cause severe testicular damage and infertility in male rats following exposure to single oral doses. There is also some evidence to suggest that infertility resulting from a single oral exposure may not be readily reversible, thereby producing permanent testicular damage. Reduced testicular weight, tubular atrophy, disruption of germinal epithelium, loss of spermatocytes, Sertoli cell damage and infertility have been reported in rats administered single oral doses of 15-50 mg/kg (Blackburn et al., 1988; Holloway et al., 1990). Significant testicular damage and reduction in fertilizing ability have also been seen in rats receiving single oral doses of 5 mg/kg (Holloway et al., 1990). Infertility was still evident in 62% of rats administered single oral doses of 48 mg 1,3-DNB, 175 days after treatment (Linder et al., 1988, 1990). The chemical appears to act on the testes

directly with the primary target of toxic action being the Sertoli cell which functions to sustain and protect developing germ cells and regulate spermatogenesis (Holloway et al., 1990). A single oral dose of 32 mg/kg did not change serum hypothalamic or pituitary hormone levels, indicating that 1,3-DNB does not effect hormonal control of gonadal function (Rehnberg et al., 1988). Work by Williams and Foster (1989) indicated that 1,3-DNB also acts independently of follicle-stimulating hormone/cAMP within the Sertoli cell. Age may to some extent affect the reproductive toxicity of 1,3-DNB, although systemic toxicity was also more pronounced in older animals, suggesting a possible reduction in intestinal metabolism and a subsequent increase in the absorption of parent compound in older animals (Linder et al., 1990; Evenson et al., 1989a). In addition, rats were found to be more sensitive to 1,3-DNB toxicity than mice with testicular damage occurring at lower doses in rats (Evenson et al., 1989a,b). 1,3-DNB or a metabolite appears to cause Sertoli cell vacuolization resulting in the exfoliation of germinal cells from the seminiferous epithelium, ultimately resulting in disruption of spermatogenesis and infertility due to lack of development of mature sperm (Foster et al., 1986; Foster, 1989; Evenson et al., 1989b; Linder et al., 1986, 1988, 1990). Thus, the action of 1,3-DNB appears to affect several stages of spermatogenesis by disrupting the morphology and function of Sertoli cells, but does not appear to affect mature sperm within the epididymal lumen (Foster, 1989).

1,3-DNB has been shown to be metabolized by cultured Sertoli cells and two major metabolites, m-nitroaniline and m-nitroacetanilide, have been identified (Foster et al., 1987). The toxicity of these metabolites, as well as several other proposed metabolites, including 2,4-dinitrophenol, 2-amino-4-nitrophenol, 4-amino-2-nitrophenol, and 2,4-diaminophenol, was compared to that of 1,3-DNB using Sertoli-germ cell co-cultures. Results indicated that none of the metabolites tested had any effect on Sertoli cell function or morphology, indicating that 1,3-DNB can be detoxified at the target site by nitroreduction. However, another minor metabolite, m-nitrosonitrobenzene (NNB), which is proposed as an intermediate in the nitroreduction of 1,3-DNB, (Foster et al., 1987; Foster, 1989; Cave and

Foster, 1990), produced the same specific toxicity effects, but at lower doses than 1,3-DNB, suggesting that 1,3-DNB may be metabolically activated and that NNB or a metabolite of NNB is the reactive intermediate in Sertoli cell toxicity.

Although 1,3-DNB has been shown by several investigators to be a potent testicular toxicant, no information on the reproductive toxicity of 1,3-DNB in females was found, and no adequate multiple-generation reproduction studies or developmental toxicity studies were available. Considering the devastating effect observed in males, evaluation of these parameters is essential to definitively assess the reproductive and developmental toxicity of 1,3-DNB.

1,3-DNB was found to be mutagenic in Salmonella typhimurium strains TA98 and TA100 (Spanggord et al., 1982; McGregor et al., 1980; Kawai et al. 1987), and in TA3052 and TA1538 (Spanggord et al., 1982; McGregor et al., 1980; Garner and Nutman, 1977) with and without metabolic activation. 1,3-DNB was not mutagenic in TA1535, TA1537, and TA100 NR3 with and without metabolic activation (Spanggord et al., 1982). In addition, 1,3-DNB did not increase unscheduled DNA synthesis in cultivated hepatocytes (Probst et al., 1981). A toxicity study in L-cell cultures determined that 1,3-DNB reduces both protein and DNA content (Cody et al., 1981). Since L-cell cultures lack hydrocarbon hydroxylase and may lack other microsomal enzymes, the toxicity in the cell culture may indicate that the parent compound is responsible for damage to the testes.

Based on these findings and on the results of a 16-week oral toxicity study in rats in which increases in spleen weight with hemosiderosis occurred at 1.14 mg/kg and decreases in testicular weight accompanied by alterations in spermatogenesis occurred at 2.64 mg/kg/day, but no effects occurred at 0.4 mg/kg/day, the Longer-term Health Advisory (HA) for a 10-kg child has been determined to be 0.04 mg/L (40 µg/L). In the absence of adequate animal data to determine a One-day or Ten-day Health Advisory, the Longer-term HA for a 10-kg child, 0.04 mg/L (40 µg/L), is used as a conservative estimate of the One-day or Ten-day HA. The Longer-term HA for an adult was established at

0.14 mg/L (140 µg/L). A Lifetime HA of 0.001 mg/L (1.0 µg/L) for an adult was determined based on a Drinking Water Equivalent Level (DWEL) of 0.005 mg/L (5 µg/L). The DWEL is based on a Reference Dose (RfD) of 0.00013 mg/kg/day, using the same 16-week study as described above for calculation of the Lifetime HA value.

C. DISCUSSION

Available data on the pharmacokinetics, health effects, analysis, and treatment of 1,3-DNB have been reviewed.

Pharmacokinetic studies in rats and rabbits indicate that 1,3-DNB is effectively absorbed via the oral route, metabolized, and excreted. Although dermal absorption is an important route of exposure, no studies yielding quantitative measurements were found in the available literature. Absorption via both the dermal and inhalation routes was assessed in a qualitative manner only; additional studies need to be conducted in animals and humans.

Available studies in humans indicate that the toxic effects produced by 1,3-DNB are consistent with those seen in animal studies; however, no exposure data were available. The oral LD_{50} in rats was calculated in one study; however no LD_{50} values were determined for other routes and in other species. Acute studies used oral or ip administration in rats; studies in other species were not found. No NOAEL or LOAEL was established in these studies; therefore, they were not suitable for development of the One- and Ten-day HA. No studies were available that reported the effects of 1,3-DNB on primary irritation and sensitization. Three subchronic studies (8, 12, and 16 weeks) using oral administration in rats were available. A NOAEL and LOAEL were established in the 16-week study for various endpoints: increased spleen weight, hemosiderin deposition in the spleen, histologic effects on the spleen, decreased testicular weight, histologic effects on the testis, and alterations in spermatogenesis. A LOAEL was established in the 12-week study based on a dose-related incidence of testicular damage and significant reductions in litter size. No NOAEL or LOAEL could be determined in the

8-week study because of significant adverse effects. No lifetime studies were found in the available literature. No subchronic studies were found in the available literature for species other than rats or for routes other than oral.

Nine studies of effects on male reproductive dysfunction, seven using a single oral dose and two using repeated oral administration for 5 days or 12 weeks, were corroborated by the effects described in the subchronic studies. The LOAEL established in the 12-week study was supported by the NOAEL and LOAEL derived from the 16-week subchronic study. A NOAEL and LOAEL were identified in one study using a single oral dose. Four studies using Sertoli-germ cell co-cultures identified the Sertoli cells as the prime target of 1,3-DNB toxicity, supporting the findings in the *in vivo* studies. No reproductive or developmental toxicity studies or data evaluating the reproductive toxicity in females were found in the available literature.

No information was found in the available literature assessing the carcinogenic potential of 1,3-DNB.

Several mutagenicity studies in *S. typhimurium* were available. An unscheduled DNA synthesis assay was also available. A toxicity assay in mouse connective tissue L cells was reported. A mutagenicity test in a eukaryotic microorganism with and without activation and a test for chromosome aberrations and sister chromatid exchange would fill this data gap.

D. CONCLUSIONS/RECOMMENDATIONS

Based on the above discussion, the following conclusions/recommendations can be made:

1. The available studies on 1,3-DNB toxicity are limited for development of Health Advisories useful in dealing with the potential contaminants of drinking water.

2. It is recommended that a 2-generation reproduction study be performed in rats, the more sensitive species.

3. It is recommended that an *in vitro* fertility assessment of Sertoli-germ cell co-cultures from rats aged 105 days or older be performed.

4. It is recommended that chronic toxicity/carcinogenicity studies be performed in rats and mice.

5. It is recommended that additional acute (primary irritation and sensitization) and subchronic studies be performed in at least one rodent species.

6. It is recommended that adequate developmental toxicity studies be performed in at least one rodent species.

7. It is recommended that single- and multiple-dose delayed neuropathy studies be performed in hens (sensitive animal model).

8. It is recommended that adequate genotoxicity studies be performed on microbial and nonmicrobial (chromosomal aberrations) cell systems.

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